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Effects of Volatile Anesthetics on Acetylcholine-induced Relaxation in the Rabbit Mesenteric Resistance Artery

Takashi Akata, M.D., Ph.D., Mikio Nakashima, M.D., Ph.D.,† Kenji Kodama, M.D., Ph.D.,‡ Walter A. Boyle III, M.D.,§ Shosuke Takahashi, M.D., Ph.D.‖*

Background: **Vascular endothelium plays an important role in the regulation of vascular tone. Volatile anesthetics have been shown to attenuate endothelium-mediated relaxation in conductance arteries, such as aorta. However, significant differences in volatile anesthetic pharmacology between these large vessels and the small vessels that regulate systemic vascular resistance and blood flow have been documented, yet little is known about volatile anesthetic action on endothelial function in resistance arteries. Furthermore, endothelium-dependent relaxation mediated by factors other than endothelium-derived relaxing factor (EDRF) has recently been recognized, and there is no information available regarding volatile anesthetic action on non-EDRF-mediated endothelium-dependent relaxation.**

Methods: **Employing isometric tension recording and microelectrode methods, the authors first characterized the endothelium-dependent relaxing and hyperpolarizing actions of acetylcholine (ACh) in rabbit small mesenteric arteries, and tested the sensitivities of these actions to EDRF pathway inhibitors and K^+ channel blockers. They then examined the effects of the volatile anesthetics isoflurane, enflurane, and sevoflurane on ACh-induced endothelium-dependent relaxation that was sensitive to EDRF inhibitors and that which was resistant to the EDRF inhibitors but sensitive to blockers of ACh-induced hyperpolarization. The effects of the volatile anesthetics on endothelium-independent sodium nitroprusside (SNP)-induced relaxation were also studied.**

Results: **Acetylcholine concentration-dependently caused both endothelium-dependent relaxation and hyperpolarization of vascular smooth muscle. The relaxation elicited by low concentrations of ACh (≤ 0.1 μM) was almost completely abolished by the EDRF inhibitors N^G-nitro L-arginine (LNNA), oxyhemoglobin (HbO_2), and methylene blue (MB). The relaxation elicited by higher concentrations of ACh (≥ 0.3 μM) was only attenuated by the EDRF inhibitors. The remaining relaxation, as well as the ACh-induced hyperpolarization that was also resistant to EDRF inhibitors, were both specifically blocked by tetraethylammonium (TEA $\geq$ 10 mM). Sodium nitroprusside, a NO donor, produced dose-dependent relaxation, but not hyperpolarization, in the endothelium-denuded (E[−]) strips, and the relaxation was inhibited by MB and HbO_2, but not TEA (≥10 mM). One MAC isoflurane, enflurane, and sevoflurane inhibited both ACh relaxation that was sensitive to the EDRF inhibitors and the ACh relaxation resistant to the EDRF inhibitors and sensitive to TEA, but not SNP relaxation (in the E[−] strips). An additional finding was that the anesthetics all significantly inhibited norepinephrine (NE) contractions in the presence and absence of the endothelium or after exposure to the EDRF inhibitors.**

Conclusions: **The results confirm that ACh has a hyperpolarizing action in rabbit small mesenteric resistance arteries that is independent of EDRF inhibitors but blocked by the K^+ channel blocker TEA. The ACh relaxation in these resistance arteries thus appears to consist of distinct EDRF-mediated and hyperpolarization-mediated components. Isoflurane, enflurane, and sevoflurane inhibited both components of the ACh-induced relaxation in these small arteries, indicating a more**

* Assistant Professor, Department of Anesthesiology and Critical Care Medicine, Kyushu University.

† Assistant Professor, Department of Anesthesiology, Saga Medical School.

‡ Associate Professor, Department of Surgical Operating Center, Kyushu University.

§ Assistant Professor, Departments of Anesthesiology, Molecular Biology and Pharmacology, Washington University.

‖ Chairman and Professor, Department of Anesthesiology and Critical Care Medicine, Kyushu University.

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Address reprint requests to Dr. Akata: Department of Anesthesiology, Research Unit, Washington University School of Medicine, Campus Box 8054, 660 South Euclid Avenue, St. Louis, Missouri 63110-1093 (until October 31, 1995) or Department of Anesthesiology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan (as of November 1, 1995).

global depression of endothelial function or ACh signaling in endothelial cells, rather than a specific effect on the EDRF pathway. All these anesthetics exerted vasodilating action in the presence of NE, the primary neurotransmitter of the sympathetic nervous system, which plays a major role in maintaining vasomotor tone *in vivo*. This strongly indicates that the vasodilating action of these anesthetics probably dominates over their inhibitory action on the EDRF pathway and, presumably, contributes to their known hypotensive effects *in vivo*. Finally, the vasodilating action of these anesthetics is, at least in part, independent from endothelium. (Key words: Anesthetics, volatile: enflurane; isoflurane; sevoflurane. Endothelium: endothelium-derived hyperpolarizing factor; endothelium-derived relaxing factor; nitric oxide. Muscle, smooth: vascular.)

VASCULAR endothelium appears to play a crucial role in the regulation of vascular smooth muscle tone by releasing vasoactive substances, such as endothelium-derived relaxing factor (EDRF), prostacyclin, or endothelin, in response to various stimuli. It has been suggested that volatile anesthetics, including halothane, enflurane, isoflurane, and sevoflurane, may significantly affect such endothelial function.[1–4] Muldoon *et al.* first reported that halothane attenuated endothelium-mediated vasorelaxation induced by acetylcholine (ACh) or bradykinin in rabbit aorta and canine femoral and carotid arteries, while endothelium-independent relaxation induced by nitroglycerin (NTG) was not affected.[1] These findings have subsequently been confirmed by Uggeri *et al.*, who studied enflurane and isoflurane, in addition to halothane, and reported that all three anesthetics attenuated both receptor- and non-receptor-mediated endothelium-dependent relaxations in rat aorta without affecting endothelium-independent relaxation induced by sodium nitroprusside (SNP).[2] Similarly, Toda *et al.* recently reported that halothane and isoflurane inhibited ACh-induced, but not NTG- or SNP-induced, relaxation in rat aorta,[4] and the lack of effect of halothane on SNP-induced relaxation was more recently confirmed by Blaise *et al.* in rabbit aorta.[5] Although these findings have led to the belief that halothane and other volatile anesthetics may inhibit the synthesis, release, or both of EDRF or inactivate EDRF, Hart *et al.* have recently demonstrated that halothane inhibited the relaxation and cyclic GMP production induced by nitric oxide (NO)—believed to be identical to EDRF—in endothelium-denuded strips from rat aorta.[6] Thus, halothane-induced attenuation of endothelium-mediated relaxation may also involve inhibition of guanylate cyclase in vascular smooth muscle cells.

Although various volatile anesthetics have been reported to attenuate endothelium-dependent relaxation in large arteries, such as rabbit or rat aorta, as described above, little information is available regarding the effects of the volatile anesthetics on endothelium-mediated vasorelaxation in small resistance arteries. It is well established that these small arteries and arterioles play an important role in the regulation of local blood flow and systemic arterial pressure.[7,8] Understanding direct anesthetic actions on endothelial function in these vessels may, therefore, prove important in understanding the cardiovascular actions of these agents. Furthermore, previous studies, both *in vivo* and *in vitro*, have documented that there exist significant differences in volatile anesthetic pharmacology between conductance and resistance arteries,[9–14] indicating the need to further investigate volatile anesthetic action in small resistance arteries.

Endothelium-dependent relaxation that is resistant to EDRF inhibitors has recently been reported,[15–18] and such relaxation may be mediated by an endothelium-derived hyperpolarizing factor (EDHF)[19–24] that is distinct from EDRF.[15,19,23,24] However, although the relative importance of EDRF and EDHF in the endothelium-dependent relaxation in various types of vessels is under active investigation,[25] there is, as yet, no information regarding a difference in volatile anesthetic actions on endothelium-dependent relaxation that is sensitive or resistant to EDRF inhibitors.

In this report, we describe the actions of the volatile anesthetics isoflurane, enflurane, and sevoflurane on endothelium-dependent relaxation in small rabbit mesenteric resistance vessels. Employing isometric tension recording and microelectrode methods, we first characterized the vasorelaxing and membrane-hyperpolarizing actions of ACh and SNP, and the sensitivity of the ACh and SNP responses to EDRF inhibitors. After this, we examined the effects of isoflurane, enflurane, and sevoflurane on the endothelium-dependent ACh-induced relaxations that were sensitive and resistant to the EDRF inhibitors, and the effects of these anesthetics on the endothelium-independent relaxation produced by SNP.

Materials and Methods

Tissue Preparation

After receiving institutional approval for this animal study, male albino rabbits (2–2.5 kg) were given so-

dium pentobarbital (40 mg/kg intravenously) and exsanguinated. The mesentery in the jejunal region was immediately placed in a dissecting chamber filled with preoxygenated Krebs-bicarbonate solution, and the mesenteric artery was rapidly excised. The distal portion of the third order branch (0.2–0.3 mm in diameter; the main truncus was not counted) were used for the current experiments. Under a binocular microscope, the fat and adhering connective tissue were carefully removed, and thin circumferential strips (0.4–0.6 mm long and 0.1–0.12 mm wide) were prepared for tension-recording. The length and diameter of the preparations for the electrophysiologic experiment were about 10 and 0.2 mm (without dissection), respectively. In some experiments, the endothelium was removed by gently rubbing the intimal surface with the round surface of a small pin, as previously described.[26] Each vascular preparation was obtained from a different animal in both the tension-recording and electrophysiologic experiments.

Recording of Mechanical Activity and Membrane Potential Measurements

Mechanical responses were measured by attaching the strips to a strain gauge (UL-2 type, Shinko Co., Tokyo) in a small chamber (0.9 ml capacity) filled with Krebs solution, as previously reported.[17,26,27] The chamber was placed on a microscope stage and the strip was stretched to 1.1 times its resting (without tension) length to obtain maximal contractile response to high K^+. The protocol for the experiments to determine the optimal stretch was as follows. After a 30-min equilibration time, high K^+ (40 mM) was applied to the strip (without stretch) for 2 min with an interval of 8 min to allow the contractile response to high K^+ to become constant. The length was then cumulatively increased in a stepwise fashion, and the contractile response was allowed to stabilize. Solution changes were accomplished by rapid perfusion from one end while aspirating simultaneously from the other end. All tension experiments were performed at 35°C.

Electrophysiologic experiments were performed separately from tension experiments. The membrane potential of the smooth muscle cell was measured using a glass capillary microelectrode filled with 3 M KCl (tip resistance, ≈ 40–80 MΩ), as previously reported.[15,20,26,27] The tissue segment was pinned onto a rubber plate in a chamber (1.5 ml volume) with a stretch of 30% and superfused with Krebs solution (35°C) at a flow rate of 3 ml/min. The electrode was inserted into the muscle cell from the outer surface of the vessel. Although, ideally, the vessel should be stretched to the same degree as that in tension experiments, it was impossible to impale the microelectrode without a stretch of this degree (≈30%). With the 30% stretch, the contractile response to high K^+ was ≈90% of that obtained with a stretch of 10%.

Application of agonists was initiated after a 30-min equilibration after mounting of the vascular tissues in the chambers. The experiments themselves were started after the vascular responses to norepinephrine (NE), ACh, or SNP all became constant (≈2–3 h and ≈1 h after mounting the strips in endothelium-intact and endothelium-denuded strips, respectively). Constant vascular responses to NE, ACh, or SNP were then observed for ≈3–6 h. In tension-recording experiments, only one experiment was performed in each strip; in electrophysiologic experiments, several experiments were performed when the effects of inhibitors on the ACh-induced hyperpolarization were reversible.

Solutions and Drugs

The composition of the Krebs solution was as follows (mM): NaCl 111.9, KCl 3.7, $MgCl_2$ 1.2, $CaCl_2$ 2.6, $NaHCO_3$ 25.5, KH_3PO 1.2, glucose 11.4. Solutions with various concentrations (1.2–77 mM) of K^+ were prepared by replacing NaCl with KCl, isoosmotically. The solution was bubbled with 95% O_2/5% CO_2 (*p*H 7.3–7.4, 35°C).

Norepinephrine HCl, methemoglobin (bovine), methylene blue (MB), N^G-nitro L-arginine (LNNA), glibenclamide, and apamin were obtained from Sigma Chemical Co. (St. Louis, MO). Tetraethylammonium chloride, barium chloride, 4-aminopyridine, and SNP were obtained from Nacalai Tesque (Kyoto, Japan). Isoflurane and enflurane were obtained from Dainabot Co. (Tokyo, Japan), and sevoflurane was obtained from Kodama Pharmaceutical Co. (Osaka, Japan).

Oxyhemoglobin (HbO_2) was prepared by reducing commercial bovine Hb containing 75% methemoglobin. After adding 10 times sodium dithionite to Hb (1 mM), dialysis by 200 times distilled water was carried out at 4°C. The identification and final concentration of oxyhemoglobin were determined spectrophotometrically.

Characterization of ACh and SNP Responses

The effects of endothelial removal, various concentrations of extracellular potassium (1.2–80 mM), EDRF pathway inhibitors (LNNA, MB, and HbO_2), and various

K^+ channel blockers (Glibenclamide, $BaCl_2$, 4-aminopyridine [4AP], apamin, and tetraethylammonium [TEA]) on the ACh-induced hyperpolarization and relaxation were studied. The ACh-induced hyperpolarizations were studied in the presence and absence of NE (10 μM). In tension-recording experiments, the strips were precontracted with NE, the primary neurotransmitter of sympathetic vasoconstrictor system, which plays a significant role in maintaining vasomotor tone *in vivo*. After the contractile response to NE reached a steady state, various concentrations (0.03–10 μM) of ACh were applied to the strip in a random sequence either in the presence or absence of inhibitors. In experiments using the EDRF pathway inhibitors, the strips were pretreated with the inhibitor (s) for 20 min before application of ACh or NE, and the inhibitors were then applied throughout the remainder of the experiment. Because preliminary experiments showed that indomethacin (3 μM), a cyclooxygenase inhibitor, did not significantly affect the ACh relaxation in this artery (n = 5, not shown), subsequent experiments were performed in the absence of indomethacin. In addition, because preliminary experiments indicated that ACh exerted its maximal hyperpolarizing effects within 1 min, ACh was applied for 1 min in the electrophysiologic experiments described here.

The concentrations of the EDRF inhibitors used in these experiments were sufficient to exert their maximal effects or were the maximum possible concentrations that could be used without producing significant toxicity. The maximal concentrations of LNNA and HbO_2 used, 100 μM and 10 μM, respectively, produced no more inhibition of ACh relaxation compared with 30 μM LNNA or 3 μM HbO_2 (analyzed by a two-factor [concentration, treatment] ANOVA, not shown). In contrast, 10 μM MB produced significant additional inhibition compared with 3 μM MB ($P < 0.05$ by a two-factor ANOVA), but 30 μM MB was apparently toxic to the vascular tissues, as evidenced by deterioration of contractile function that occurred ≈60–90 min after application of this concentration (30 μM) of MB.

The K^+ channel blockers tested in this study were glibenclamide (3 and 10 μM), $BaCl_2$ (30 and 100 μM), 4AP (30 and 100 μM), apamin (0.1 and 0.3 μM), and TEA (0.1–30 mM). Glibenclamide and apamine are known to selectively inhibit the ATP-sensitive K^+ channel and the low conductance Ca^{2+}-activated K^+ channel, respectively, while Ba^{2+}, 4AP, and TEA have been reported to block various types of K^+ channels, including voltage-dependent K^+ channels, Ca^{2+}-activated K^+ channels, or ATP-sensitive K^+ channels.[28]

The effect of SNP on resting membrane potential was studied in endothelium denuded (E[−]) strips. The relaxing effects of SNP, as well as the effects of MB (10 μM), HbO_2 (10 μM), and TEA (10 and 30 mM) on the SNP (1–10 μM)-induced relaxation were studied in E(−) strips preconstricted with NE (10 μM).

Anesthetic Actions

The effects of 1 MAC isoflurane (2% in rabbit), enflurane (2.8%), and sevoflurane (3.7%) on ACh-induced relaxation in endothelium intact (E[+]) strips and on SNP-induced relaxation in E(−) strips were studied.[29,30] The effects of the anesthetics on ACh and SNP relaxations were studied in strips preconstricted with NE and the anesthetics were applied for 5 min before, and then continuously during, application of NE and either NE + ACh or NE + SNP. As noted below, all three anesthetics significantly inhibited the contractions induced by 10 μM NE. To compensate for this effect, the NE concentration was increased to produce approximately the same level of tension (*i.e.*, contraction) as 10 μM NE produced in control strips (*i.e.*, without anesthetic) before measuring relaxation produced by ACh or SNP. This technique of compensating for vasodilating effects has previously been employed for both volatile anesthetics and basic polypeptides when examining the effects of these drugs on ACh relaxation.[6,31]

Volatile Anesthetics Delivery and Analysis

The volatile anesthetics were delivered *via* calibrated agent-specific vaporizers in line with the O_2/CO_2 equilibration gas mixture aerating the Krebs solution. The anesthetic concentration in the resulting gas mixture was monitored with a calibrated infrared multianesthetic gas analyzer (Capnomac; Datex, Tewksbury, MA). Preliminary experiments indicated that 10 min was sufficient for the Krebs solution to be saturated with the anesthetics at each partial pressure. Therefore, the Krebs solution was equilibrated with the anesthetics for 15 min before the study. The experimental chamber was covered with a thin glass plate to prevent the equilibration gas from escaping into the atmosphere. The concentrations of isoflurane, enflurane, and sevoflurane in the Krebs solution were also determined by gas chromatography yielding values of 0.37 ± 0.01 mM, 0.71 ± 0.05 mM, and 0.42 ± 0.05 mM, respectively (mean ± SD, n = 3–5). These values were 86, 88, and 84% of the theoretical values predicted by the partition

coefficient of isoflurane and enflurane in Krebs solution[32] or by the partition coefficient of sevoflurane in water (the partition coefficient of sevoflurane in Krebs solution is not available). Thus, these concentrations were equivalent to 0.86, 0.88, and 0.84 MAC in rabbit, respectively, although the concentration of sevoflurane may be slightly underestimated, because the partition coefficients of other volatile anesthetics in Krebs solution is about 90% of that in water.[32]

Calculation and Statistical Analysis

All results were expressed as mean ± SD. In the tension-recording experiments, n denotes the number of animals. In the electrophysiologic experiments, n denotes the number of impalements (cells); the number of preparations (animals) is noted separately. The ACh relaxation in the presence of NE and the ACh hyperpolarization were assessed at the points at which its relaxing or hyperpolarizing effects reached a maximum. The statistical assessment of the data was made by an ANOVA (one or two factor), Scheffé's F test, and Student's *t* test, as appropriate. A level of $P < 0.05$ was considered significant.

Results

Acetylcholine- and Sodium Nitroprusside-Induced Changes in Membrane Potential in Vascular Smooth Muscle

In E(+) strips, ACh hyperpolarized the membrane potential (MP) of smooth muscle cells in both the absence and the presence of NE (3 and 10 μM) (fig. 1). Norepinephrine (3 and 10 μM) itself significantly depolarized the MP, and ACh (3 μM) reversed the NE-induced depolarization in E(+) strips (fig. 1). Acetylcholine (up to 10 μM) had no significant effect on MP in E(−) strips in the absence (n = 12, five preparations) or in the presence (n = 15, five preparations) of NE (not shown).

As shown in figure 2A, 0.1–10 μM ACh hyperpolarized the resting MP of the smooth muscle cells, in both 3.6 and 5.9 mM extracellular K^+ ($[K^+]_o$), in a concentration-dependent manner. The hyperpolarizations in the presence of 3.6 mM $[K^+]_o$ were consistently larger ($P < 0.05$) than those in the presence of 5.9 mM $[K^+]_o$ (fig. 2B).

Figure 2C shows that the hyperpolarization induced by 3 μM ACh varies as a function of extracellular K^+ concentration. At low $[K^+]_o$ values, MP deviated significantly from the equilibrium potential for K^+ (E_K) predicted by the Nernst's equation, and the ACh-induced hyperpolarizations were the largest. Acetylcholine-induced hyperpolarizations almost disappeared in the presence of $[K^+]_o$ above 10 mM where MP was essentially equal to E_K.

To obtain more distinct hyperpolarizing responses to ACh, subsequent experiments using EDRF and K^+ channel inhibitors were performed in the presence of 3.6 mM $[K^+]_o$. Under these conditions, LNNA (100 μM) and HbO_2 (10 μM) did not significantly affect the resting MP, and ACh (3 μM) still hyperpolarized ($P < 0.05$) the smooth muscle MP in the presence of

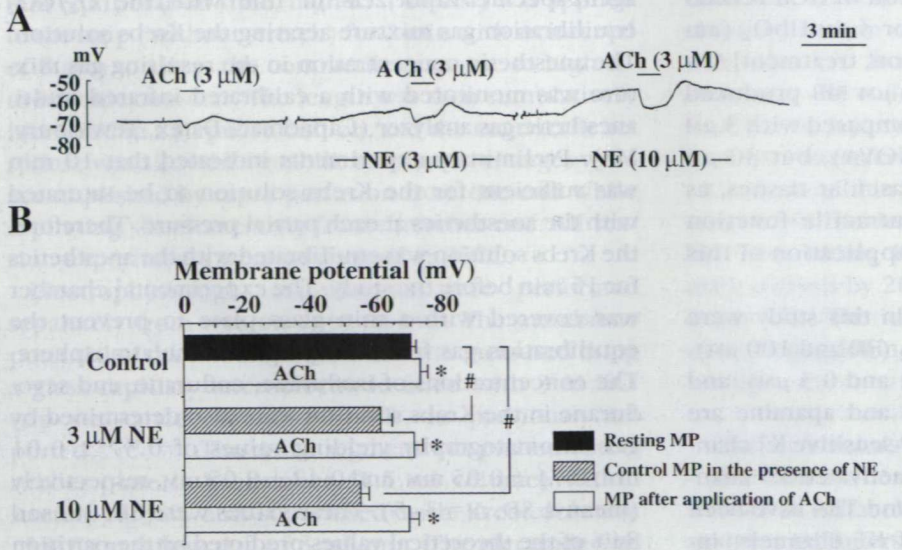

Fig. 1. Effects of ACh (3 μM) on the membrane potential in the absence and presence of NE (3 and 10 μM) in the endothelium-intact strips. (*A*) Examples of the ACh-induced hyperpolarization recorded in the same strip. (*B*) Data are mean ± SD (n = 7–15, three to five preparations). ACh = acetylcholine, MP = membrane potential, NE = norepinephrine. *Significantly different ($P < 0.05$) from the membrane potential before application of ACh. #Significantly different ($P < 0.05$) from the resting membrane potential (before application of NE).

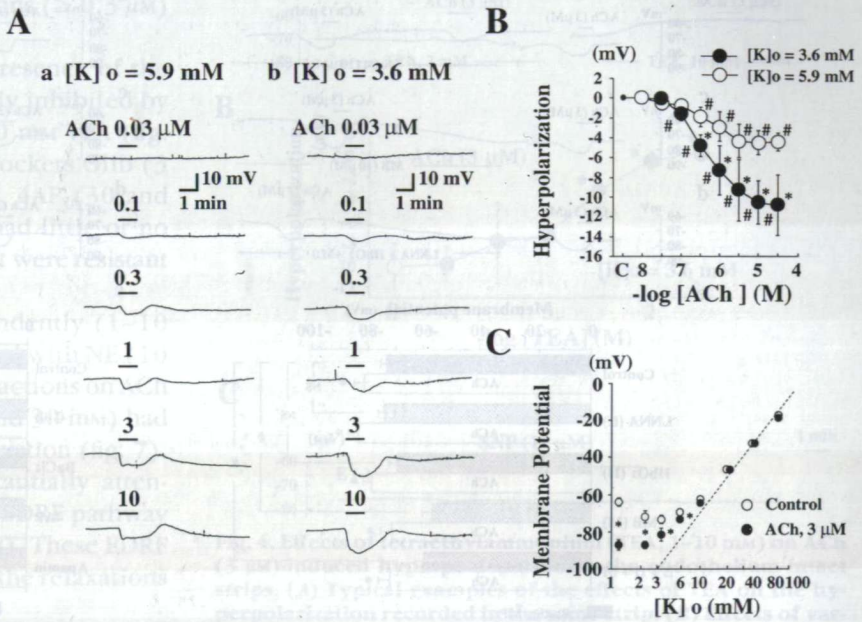

Fig. 2. Effects of ACh on the membrane potential in the presence of various extracellular K^+ concentrations in the endothelium-intact strips. (*A*) Typical examples of the ACh (0.03–10 μM)-induced hyperpolarization in the presence of 3.6 and 5.9 mM extracellular [K^+]. (*B*) The closed and open circles show the effects of ACh on the resting membrane potential in the presence of 3.6 and 5.9 mM extracellular [K^+], respectively. Data are mean ± SD (n = 5–11, four to five preparations). ACh = acetylcholine, $[K^+]_o$ = extracellular K^+ concentration. #Significantly different ($P < 0.05$) from the membrane potential before the application of ACh within each group. *Significantly different ($P < 0.05$) from the ACh-induced hyperpolarization in the presence of 5.9 mM extracellular K^+ at each concentration. (*C*) The effects of ACh (3 μM) on the membrane potential in the presence of various extracellular K^+ concentrations. The closed circles show the effects of ACh on the membrane potential in the presence of various extracellular K^+ concentrations (1.2–77 mM), whereas the open circles show the control membrane potential in the absence of ACh. The dotted line indicates the equilibrium potential for K^+ calculated from Nernst's equation (35°C), assuming that the intracellular K^+ concentration is 140 mM. Data are mean ± SD (n = 6–25, three to five preparations). $[K^+]_o$ = extracellular K^+ concentration. *Significantly different ($P < 0.05$) from the control membrane potential at each concentration.

these inhibitors (fig. 3A). The hyperpolarizations in the presence of LNNA and HbO_2 were 9.6 ± 3.6 mV (n = 8, four preparations) and 10.3 ± 3.2 mV (n = 5, three preparations), respectively, both of which were not significantly different from the control hyperpolarization measured in the absence of the inhibitors (10.5 ± 2.2 mV [n = 9, four preparations]). Methylene blue (10 μM) and a cocktail application of LNNA (100 μM), HbO_2 (10 μM), and MB (10 μM) significantly depolarized the resting MP (fig. 3A). However, the ACh-induced hyperpolarizations were at least as large as the control response after the inhibitors, and the absolute level of MP achieved after ACh was not different than control with any of the inhibitors.

The K^+ channel blockers glibenclamide (Glib, 10 μM), $BaCl_2$ (100 μM), 4-aminopyridine (4AP, 100 μM), or apamin (0.3 μM) had little if any effect on the hyperpolarizing action of ACh (fig. 3B). The hyperpolarization in the presence of apamin (9.2 ± 3.7 mV, n = 5, three preparations), which was the only K^+ blocker that did not affect MP, was not significantly different from the control hyperpolarization (10.5 ± 2.2 mV, n = 9, four preparations). Once again, the magnitude of the ACh-induced hyperpolarizations in the presence of all these K^+ channel blockers were at least as large as the control responses, and the absolute level of MP achieved after ACh was not different from control.

The K^+ channel blocker TEA (3–30 mM) also caused some depolarization in MP, but TEA substantially attenuated the ACh-induced hyperpolarization such that the ACh response was completely abolished at TEA concentrations ≥ 10 mM (fig. 4). Among all the K^+ channel and EDRF blockers tested, TEA (≥10 mM) appeared to be the only agent capable of blocking the hyperpolarizing action of ACh.

In contrast to the effects of ACh on MP, SNP (up to 10 μM) had no apparent effect on the resting MP in the E(−) strips (fig. 4C). There was no significant differ-

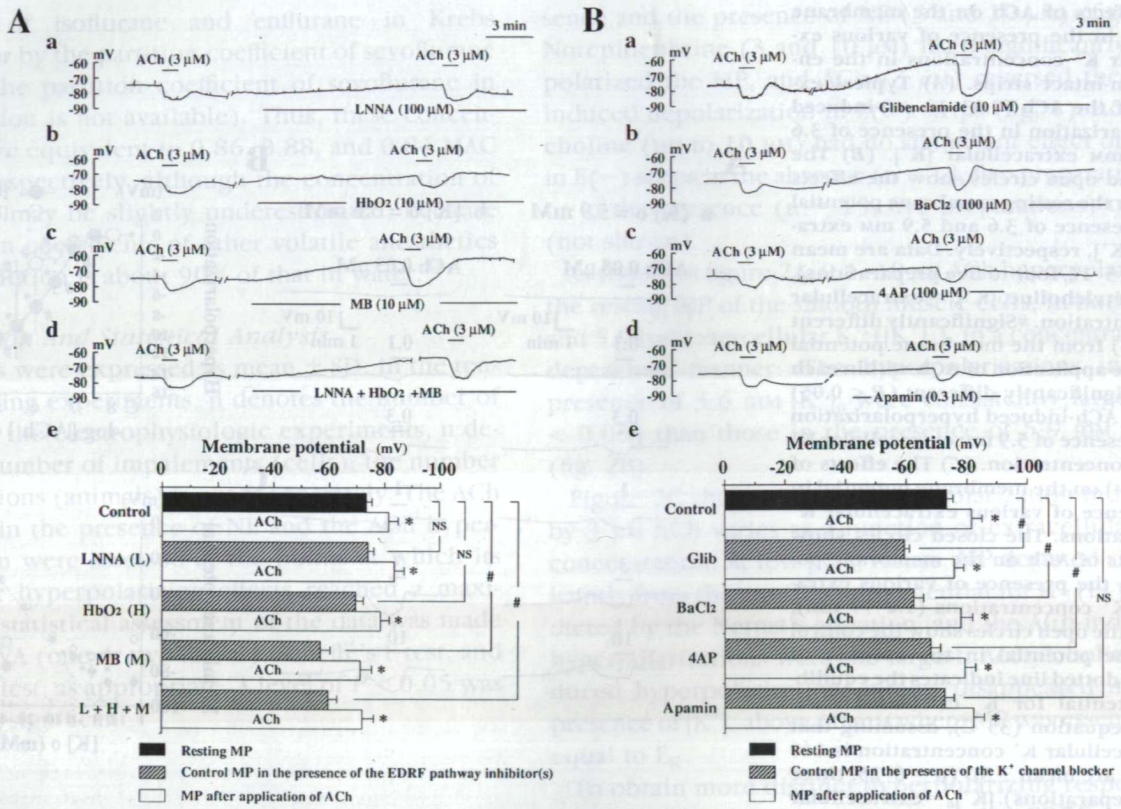

Fig. 3. Effects of various pharmacologic inhibitors on the ACh (3 μM)-induced hyperpolarization in the endothelium-intact strips. (*A*) The effects of various inhibitors of EDRF pathway on the ACh hyperpolarization. (*a–d*) Typical examples of the effects of N^G-nitro L-arginine (LNNA, 100 μM), oxyhemoglobin (HbO_2, 10 μM), methylene blue (MB, 10 μM), and a cocktail application of LNNA (100 μM), HbO_2 (10 μM), and MB (10 μM) on the hyperpolarization. (*e*) Effects of these EDRF inhibitors on the resting membrane potential and the effects of ACh on the membrane potential in the presence of the EDRF inhibitors. Data are mean ± SD (n = 5–9). *Significantly different ($P < 0.05$) from the membrane potential before application of ACh either in the absence or presence of EDRF inhibitors. #Significantly different ($P < 0.05$) from the control membrane potential before application of the EDRF inhibitors. NS = not significantly different from the control membrane potential before application of the EDRF inhibitors. (*B*) Effects of various K channel blockers on the ACh hyperpolarization. (*a–d*) Typical examples of the effects of glibenclamide (Glib, 10 μM), $BaCl_2$ (100 μM), 4-aminopyridine (4AP, 100 μM), and apamin (0.3 μM) on the hyperpolarization. (*e*) Effects of various K^+ channel blockers on the resting membrane potential and the effects of ACh on the membrane potential in the presence of these K^+ channel blockers. Data are mean ± SD (n = 5–11, three to four preparations). *Significantly different ($P < 0.05$) from the control membrane potential before application of ACh either in the absence or presence of K^+ channel blockers. #Significantly different ($P < 0.05$) from the control membrane potential before application of the K^+ channel blockers. NS = not significantly different from the control membrane potential before application of the K^+ channel blockers.

ence in the MP measured before (−73.4 ± 2.1 mV, four preparations) and after 10 μM SNP (72.9 ± 3 mV, n = 12, four preparations).

Acetylcholine- and Sodium Nitroprusside-Induced Relaxation of Norepinephrine Contracted Vascular Smooth Muscle

In E(+) strips, 0.03 to 10 μM ACh relaxed the strips precontracted with NE (10 μM) in a concentration-dependent manner (fig. 5A and D). In contrast, even very high concentrations of ACh (up to 10 μM) failed to relax E(−) strips precontracted with NE (10 μM) (not shown). The application of the EDRF pathway inhibitors LNNA (100 μM), HbO_2 (10 μM), MB (10 μM), or a cocktail application of LNNA (100 μM), HbO_2 (10 μM), and MB (10 μM) significantly enhanced the contractile response to NE in the E(+) strips: LNNA, 2.4 ± 1.8 times control (n = 31); HbO_2, 1.7 ± 0.5 times control (n = 8); MB, 2.5 ± 1.5 times control (n = 29); and LNNA + HbO_2 + MB, 3.3 ± 2.1 times control (n = 28, fig. 5B and C). These EDRF inhibitors almost abolished the relaxations elicited by the low concentrations (≤

0.1 μM) of ACh, but failed to completely abolish the relaxations elicited by high concentrations (≥ 0.3 μM) of ACh (fig. 5B–D). Even a cocktail application of LNNA, HbO_2, and MB failed to completely abolish the relaxations elicited by high concentrations (≥ 0.3 μM) of ACh (fig. 5C and D).

The residual ACh relaxation in the presence of the EDRF pathway inhibitors was completely inhibited by the additional application of 10 and 30 mM TEA (fig. 5C and D). However, the K^+ channel blockers Glib (3 and 10 μM), apamin (0.1 and 0.3 μM), 4AP (30 and 100 μM), and $BaCl_2$ (30 and 100 μM) had little or no effect on the residual ACh relaxations that were resistant to the EDRF inhibitors (fig. 6).

As shown in figure 7, SNP dose-dependently (1–10 μM) relaxed the E(−) strips precontracted with NE (10 μM) (fig. 7). However, in contrast to the actions on ACh relaxation in the E(+) strips, TEA (10 and 30 mM) had no significant effect on SNP-induced relaxation (fig. 7). The SNP-induced relaxation was substantially attenuated by the cocktail application of the EDRF pathway inhibitors HbO_2 (10 μM) and MB (10 μM). These EDRF inhibitors almost completely abolished the relaxations elicited by 1 and 3 μM SNP (fig. 7A–C).

Effects of Volatile Anesthetics on Acetylcholine- and Sodium Nitroprusside-Induced Relaxations

As shown above, the relaxations produced by the low concentrations (0.03–0.3 μM) of ACh were abolished or strongly inhibited by the EDRF inhibitors (fig. 5). Isoflurane, enflurane, and sevoflurane all caused significant and reversible inhibition of these endothelium-dependent ACh-induced relaxations that were sensitive to the EDRF pathway inhibitors, as shown in figure 8, and no significant differences were observed in their inhibitory effects on ACh relaxation among these anesthetics.

Because all three anesthetics significantly inhibited the NE (10 μM) contractions (fig. 8A–D), the concentrations of NE were increased in the strips pretreated with the anesthetics to obtain a NE precontraction with the same amplitude as that in the control. The concentrations of NE required to obtain precontractions with the same amplitude as the control were 36.7 ± 12.9 μM (n = 7), 25.6 ± 5.3 μM (n = 9), and 50.6 ± 14.1 μM (n = 6) in the presence of isoflurane, enflurane, and sevoflurane, respectively. Using these concentrations of NE, there were no significant differences in the amplitude of the NE precontractions before and after the anesthetic in all groups. There were no significant differences among the anesthetics for their inhibitory effects on precontraction induced by 10 μM NE (fig. 8D).

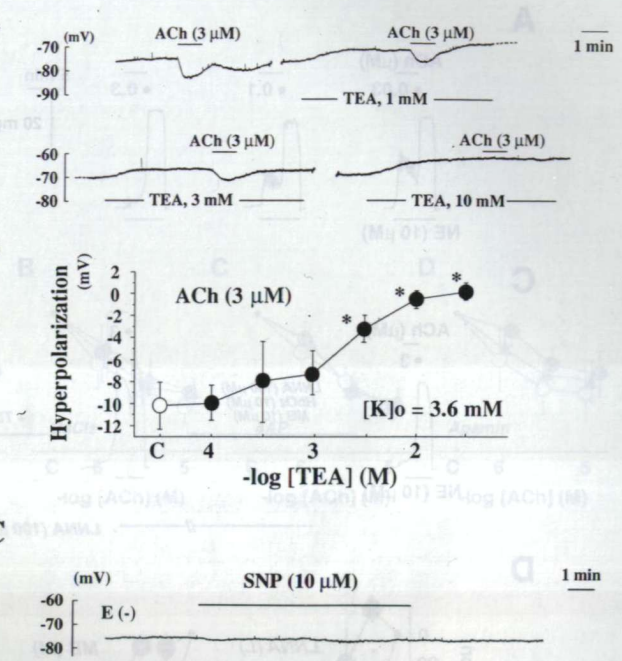

Fig. 4. Effects of tetraethylammonium (TEA, 1–10 mM) on ACh (3 μM)-induced hyperpolarization in the endothelium-intact strips. (*A*) Typical examples of the effects of TEA on the hyperpolarization recorded in the same strip. (*B*) Effects of various concentrations of TEA (0.1–30 mM) on the ACh hyperpolarization. Data are mean ± SD (n = 6–9, four preparations). *Significantly different ($P < 0.05$) from the control hyperpolarization (in the absence of TEA, open circle). (*C*) Effects of SNP (10 μM) on the resting membrane potential in the endothelium-denuded strips.

The effects of volatile anesthetics on ACh relaxation resistant to the EDRF inhibitors were studied using the relaxation induced by 3 μM ACh in the presence of the (EDRF) inhibitors. This relaxation was sensitive to the K^+ channel blocker TEA (fig. 5), which selectively blocked the endothelial-dependent hyperpolarizing action of ACh (fig. 4) that was resistant to the EDRF inhibitors (fig. 3). All three anesthetics significantly and reversibly inhibited this 3 μM ACh-induced relaxation in the presence of the EDRF inhibitors (fig. 9D), and no significant differences were observed in the magnitudes of their inhibitory effects on ACh relaxation in the presence of the EDRF inhibitors. All three anesthetics again produced significant inhibition of the precontraction induced by 10 μM NE ($P < 0.05$), and the concentrations of NE in the strips pretreated with the anesthetics were increased to obtain a NE-induced

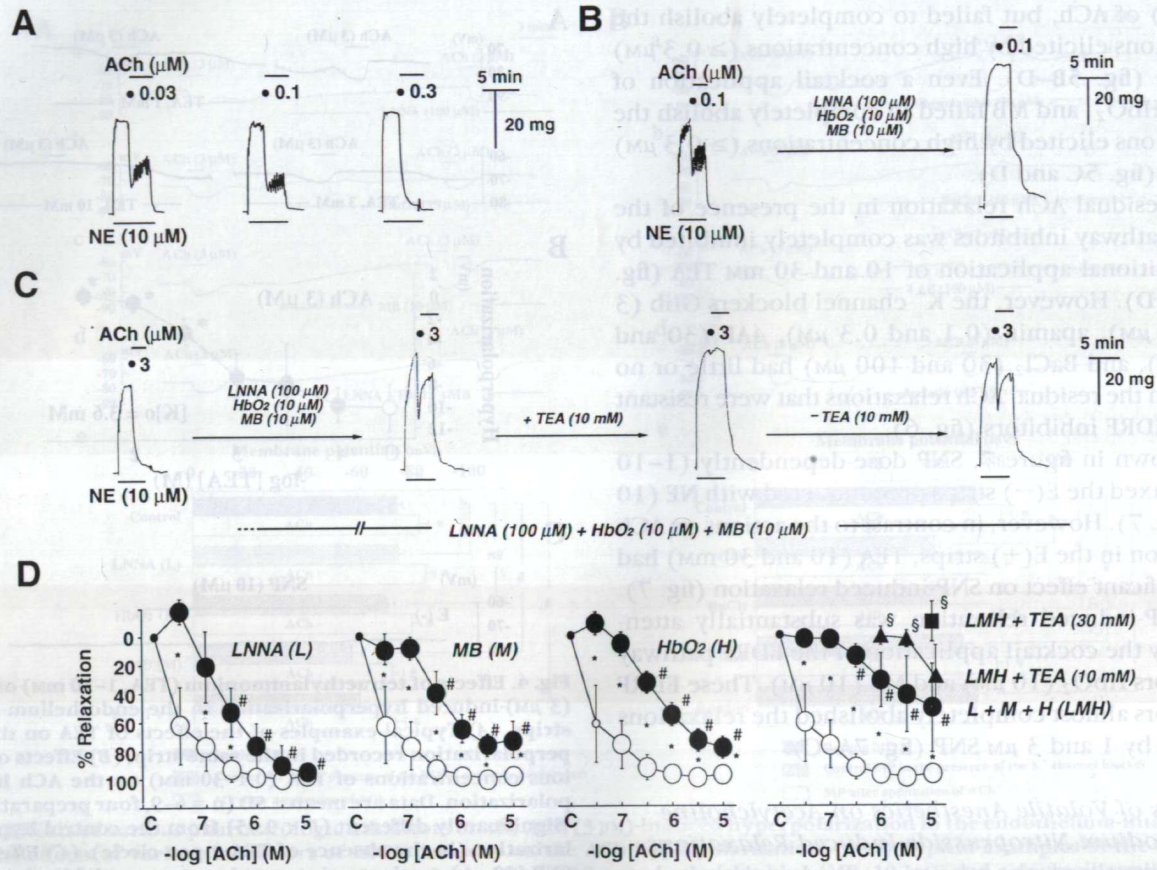

Fig. 5. The effects of various inhibitors of the EDRF pathway inhibitors and tetraethylammonium (TEA) on the ACh-induced relaxation in the endothelium-intact strips precontracted with norepinephrine (NE, 10 μM). (*A*) Examples of ACh (0.03–0.3 μM)-induced endothelium-dependent relaxation in the absence of EDRF pathway inhibitors (control). (*B*) The effects of a cocktail application of N^G-nitro L-arginine (LNNA, 100 μM), oxyhemoglobin (HbO_2, 10 μM), and methylene blue (MB, 10 μM) on the relaxation elicited by a low concentration of ACh (0.1 μM). (*C*) The effects of TEA (10 mM) on the remaining ACh (3 μM)-induced relaxation after application of the EDRF pathway inhibitors. (*D*) The effects of EDRF pathway inhibitors (100 μM LNNA [L], 10 μM MB [M], 10 μM HbO_2 [H], and a cocktail application of 100 μM LNNA, 10 μM MB, and 10 μM HbO_2 [L + M + H]) on the ACh (0.03–10 μM)-induced vasorelaxation. The open circles show the control ACh relaxation in the absence of the inhibitors (control). The closed circles show the effects of the above interventions on the ACh relaxation. C = control, L = LNNA, N^G-nitro L-arginine, M = MB, methylene blue, H = HbO_2, oxyhemoglobin, LMH = L + M + H, a cocktail application of 100 μM LNNA, 10 μM MB, and 10 μM HbO_2. All data are mean ± SD (n = 3–8). *Significantly different ($P < 0.05$) from the control ACh-induced relaxation at each concentration. #Significantly different ($P < 0.05$) from the control within each group (this symbol was not added to the control group). §Significantly different ($P < 0.05$) from the LMH group at each concentration.

precontraction of the same amplitude as in the control (fig. 9). There were no significant differences among the three anesthetics in the amount of inhibition of the 10 μM NE contraction (fig. 9E), and the concentrations of NE required to obtain a precontraction of the same amplitude as the control were 14.3 ± 4.6 (n = 6), 14.8 ± 3.5 (n = 4), and 16.5 ± 4 μM (n = 4), in the presence of isoflurane, enflurane, and sevoflurane, respectively.

The effects of volatile anesthetics on the endothelium-independent relaxation induced by SNP (3 μM) in the E(−) strips are shown in figure 10. As described above, this SNP-induced relaxation was almost completely abolished by the EDRF inhibitors MB and HbO_2 (fig. 7). In contrast to their effects on ACh-induced relaxation, however, neither isoflurane, enflurane, nor sevoflurane had any significantly inhibitory effect on the SNP (3 μM ≈ IC_{50} values)-induced relaxation (fig. 10A–D). As in the E(+) strips, the three anesthetics significantly inhibited the NE-induced precontraction in the E(−) strips (fig. 10A–C and E). The concentrations of NE in the strips pretreated with the anesthetics were

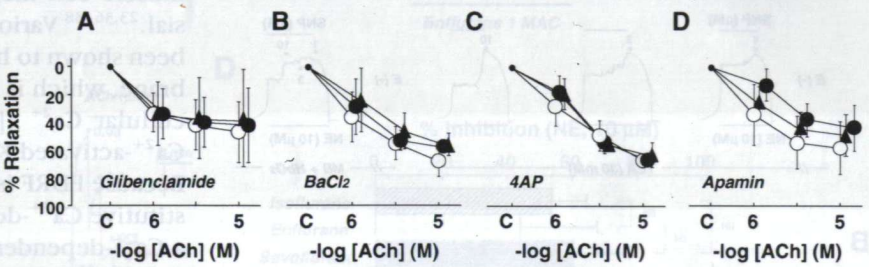

Fig. 6. Effects of various K^+ channel blockers on ACh-induced relaxation in the endothelium-intact strips precontracted with norepinephrine (NE, 10 μM) in the presence of the EDRF pathway inhibitors N^G-nitro L-arginine (LNNA, 100 μM), oxyhemoglobin (HbO_2, 10 μM) and methylene blue (MB, 10 μM). The open circles show the control ACh relaxation in the presence of these EDRF inhibitors, while the closed symbols show the effects of various K^+ channel blockers on the ACh relaxation resistant to the EDRF inhibitors: glibenclamide (*A*, closed circles, 3 μM; closed triangles, 10 μM), $BaCl_2$ (*B*, closed circles, 30 μM; closed triangles, 100 μM), 4-aminopyridine (*C*, closed circles, 30 μM; closed triangles, 100 μM), and apamin (*D*, closed circles, 0.1 μM; closed triangles, 0.3 μM). All above data are the mean ± SD (n = 3–6). These K^+ channel blockers at the concentrations tested did not significantly affect the ACh-induced relaxation in the presence of the EDRF pathway inhibitors. Note that the control ACh relaxation was recorded in the presence of EDRF inhibitors. ACh = acetylcholine, 4AP = 4-aminopyridine.

again increased to obtain almost the same NE precontraction amplitude as the control before application of SNP. The concentrations of NE required to obtain precontractions of the same amplitude as the control were 18.33 ± 4.1 (n = 6), 14.3 ± 5.3 (n = 4), and 20 ± 0 μM (n = 4), in the presence of isoflurane, enflurane, and sevoflurane, respectively. There were no significant differences in the amplitude of the NE contraction before and after the anesthetics in all three groups, and there were no significant differences in the inhibitory effects of the anesthetics on the contraction induced by 10 μM NE (fig. 10E).

One additional finding of interest was the transient constriction induced by enflurane in both the E(+) and E(−) strips (figs. 8B, 9B, and 10B). Similar transient constrictions were not observed with either isoflurane and sevoflurane (not shown). The amplitudes of the enflurane-induced contraction in the E(+) strips, in the E(+) strips pretreated with the EDRF inhibitors, and in the E(−) strips were 0.81 ± 0.20 (n = 9), 0.72 ± 0.12 (n = 4), and 0.73 ± 0.08 (n = 6) times the amplitude of a 40 mM K^+-induced phasic contraction in each condition, respectively. Furthermore, enflurane generated a similar transient contraction in Ca^{2+}-free, 2 mM EGTA solution in the E(−) strips with an amplitude equivalent to 0.75 ± 0.12 (n = 3) times that of the 40 mM K^+-induced phasic contraction (in the presence of 2.6 mM Ca^{2+}).

Discussion

Characteristics of the Acetylcholine-Induced Relaxation and Hyperpolarization

The current study demonstrated that ACh causes endothelium-dependent relaxation and hyperpolarization of the smooth muscle in this small splanchnic resistance artery, as observed previously in other arteries.[15–26] In addition, our results strongly indicate that the relaxation induced by low concentrations (≤ 0.1 μM) of ACh is totally dependent on the action of EDRF, but the relaxation induced by higher concentrations (≥ 0.3 μM) of ACh is, in part, mediated by a hyperpolarizing effect of ACh that is independent of the action of EDRF. In support of this, only the relaxation induced by the low concentrations of ACh was abolished by the inhibitors of the EDRF (NO) pathway, including LNNA, HbO_2, and MB. The concentrations of ACh that produced relaxation resistant to the EDRF inhibitors, *i.e.*, more than 0.3 μM, corresponded to those that produced hyperpolarization that was not affected by the EDRF

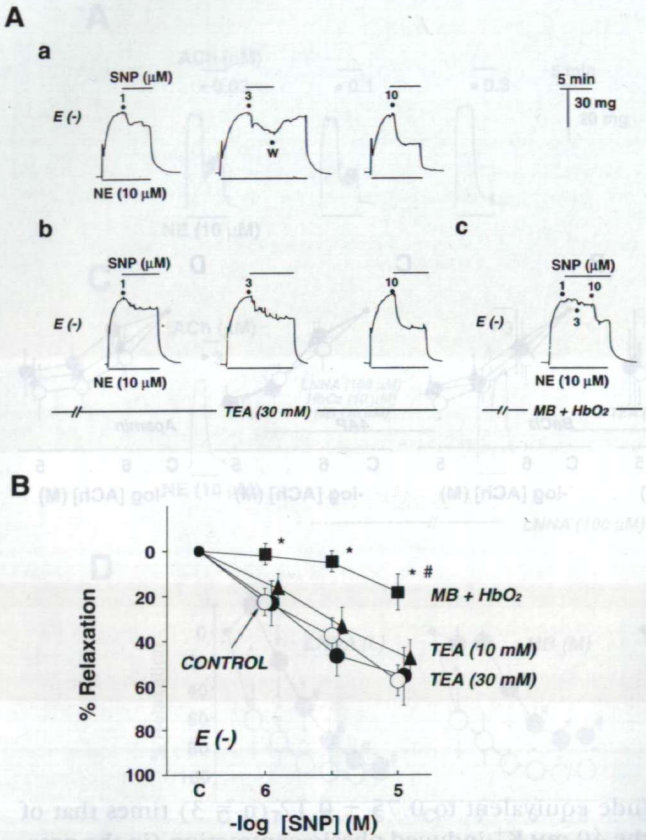

Fig. 7. The effects of tetraethylammonium (TEA, 30 mM) and a cocktail application of methylene blue (MB, 10 μM) and oxyhemoglobin (HbO_2, 10 μM) on the SNP (1–10 μM)-induced relaxation in the endothelium-denuded strips precontracted with norepinephrine (NE, 10 μM). (*A*) (1) The SNP-induced relaxation in the absence of inhibitors (control). (2) The SNP-induced relaxation in the presence of TEA (30 mM). (3) The inhibitory effects of HbO_2 and MB on the SNP-induced relaxation. (*B*) Effects of TEA, HbO_2, and MB on the SNP-induced relaxation. Data are mean ± SD (n = 3–8). *Significantly different ($P < 0.05$) from the control relaxation at each concentration. #Significantly different ($P < 0.05$) from the control within each group. W = washout of SNP.

inhibitors. Moreover, both the ACh relaxation resistant to the EDRF inhibitors and the ACh hyperpolarization were strongly inhibited or abolished by TEA (10–30 mM). Finally, the effects of SNP strongly indicate that the ACh hyperpolarization is independent from the action of EDRF. The vasorelaxation produced by SNP, which results from NO (*i.e.,* EDRF) generation in smooth muscle independent of the endothelium, was strongly inhibited by the EDRF inhibitors; however, SNP had no effect on membrane potential and SNP-induced relaxation was not affected by TEA (10–30 mM). The proposed mechanisms of the hyperpolarization-mediated vasorelaxation are inhibition of the voltage-dependent Ca^{2+} channels, inhibition of IP_3 synthesis, or both.[33–35]

Whether the hyperpolarization is mediated by some humoral substance(s), *i.e.,* EDHF, or by electrotonic spread of endothelial hyperpolarization to smooth muscle cell membrane still appears to be controversial.[23,36–38] Various EDRF releasers, including ACh, have been shown to hyperpolarize "endothelial" cell membrane, which is thought to result from increased intracellular Ca^{2+} ($[Ca^{2+}]i$) and subsequent activation of Ca^{2+}-activated K^+ channels in endothelial cells.[37,39–41] Because EDRF is synthesized from L-arginine by a constitutive Ca^{2+}-dependent NO-synthase and released by a Ca^{2+}-dependent mechanism,[40,41] the endothelial hyperpolarization has been proposed to serve as an important signal for synthesis and release of EDRF by providing the necessary driving force for transmembrane Ca^{2+} influx, and sustained increases in $[Ca^{2+}]i$ in endothelial cells. This endothelial hyperpolarization could then be transmitted to smooth muscle cells electrotonically *via* some low-resistance electrical pathway such as gap junctions, which are known to exist between endothelial and smooth muscle cells.[37,38,42,43] According to this model, the hyperpolarization of the smooth muscle membrane would thus be generated electronically as part of the signal transduction pathway involved in the synthesis and release of EDRF from endothelial cells; and TEA, a K^+ channel blocker, may block the EDRF inhibitor-resistant component of the ACh relaxation by inhibiting endothelial Ca^{2+}-activated K^+ channels, thereby preventing endothelial and smooth muscle hyperpolarization.

Alternatively, several recent studies have demonstrated that ACh can generate smooth muscle hyperpolarization in situations in which electrotonic transmission cannot occur.[21,36,44] It has thus been suggested that ACh hyperpolarization may be mediated by some endothelial-derived hyperpolarizing factor(s) (EDHF). In further support of this model, Beny has demonstrated that injection of electrical pulses into endothelial cells does not evoke any electrical response in the smooth muscle cells of rabbit aorta, indicating that electrotonic spread of hyperpolarization through gap junctions does not occur.[45] Assuming that ACh hyperpolarizations are thus mediated by some endothelial humoral substance, the lack of effect of LNNA, HbO_2, or MB on the ACh-induced hyperpolarization, as well as the lack of effect of SNP on membrane potential, indicates that EDHF is

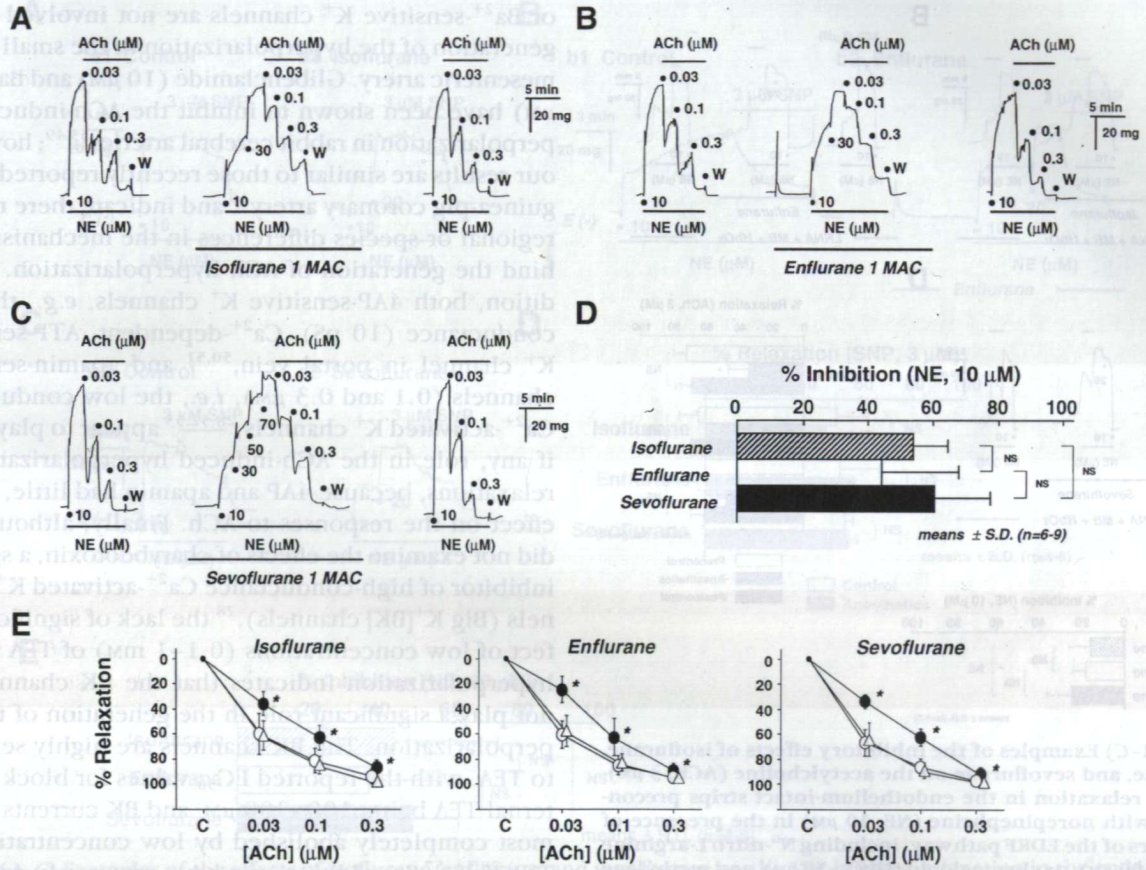

Fig. 8. Effects of isoflurane, enflurane, and sevoflurane on the relaxation elicited by low concentrations of acetylcholine (ACh) in the endothelium-intact strips precontracted with norepinephrine (NE, 10 μM). (*A–C*) Examples of the inhibitory effects of isoflurane, enflurane, and sevoflurane on the ACh (0.03–0.3 μM)-induced relaxation. The left, middle, and right panels of each figure show the ACh relaxation before exposure to the anesthetics (precontrol), in the presence of the anesthetics, and after washout of the anesthetics from the bath (postcontrol), respectively. W = washout of ACh and NE. (*D*) Inhibitory effects of isoflurane, enflurane, and sevoflurane on the NE (10 μM)-induced contractions. NS = no significant differences between the groups. (*E*) The open circles, closed circles, and open triangles show the precontrol ACh relaxation, the ACh relaxation in the presence of the anesthetics, and the postcontrol ACh relaxation, respectively. No significant difference were observed in the ACh relaxation between the precontrol and postcontrol groups in all experiments. Values are mean ± SD (n = 6–9). *$P < 0.05$ *versus* pre- and postcontrol at each concentration.

distinct from EDRF (NO). This leads to the proposal that the ACh relaxation in these small mesenteric arteries consists of both EDRF- and EDHF-mediated components. According to this model, TEA may block K^+ channels in endothelial cells that are important in the generation of the EDHF signal, or TEA may block K^+ channels in vascular smooth muscles that are involved in the EDHF hyperpolarizing response.

Methylene blue and the cocktail application of LNNA, HbO_2, and MB significantly depolarized the membrane potential. However, it is difficult to conclude from this that EDRF itself has a hyperpolarizing effect in this artery, because LNNA, which is known to be the most specific inhibitor of the EDRF pathway,[46] did not affect the membrane potential. Additionally, SNP, which presumably results in generation of NO in vascular smooth muscle, had no effect on membrane potential. The mechanism of the depolarizing effects of MB is currently unknown, but could reflect a toxic effect because higher concentrations of MB caused rapid deterioration of the preparation.

Ionic Mechanisms of the Acetylcholine-Induced Hyperpolarization

Our results strongly indicate that the ACh hyperpolarization is mediated through an increase in K^+ per-

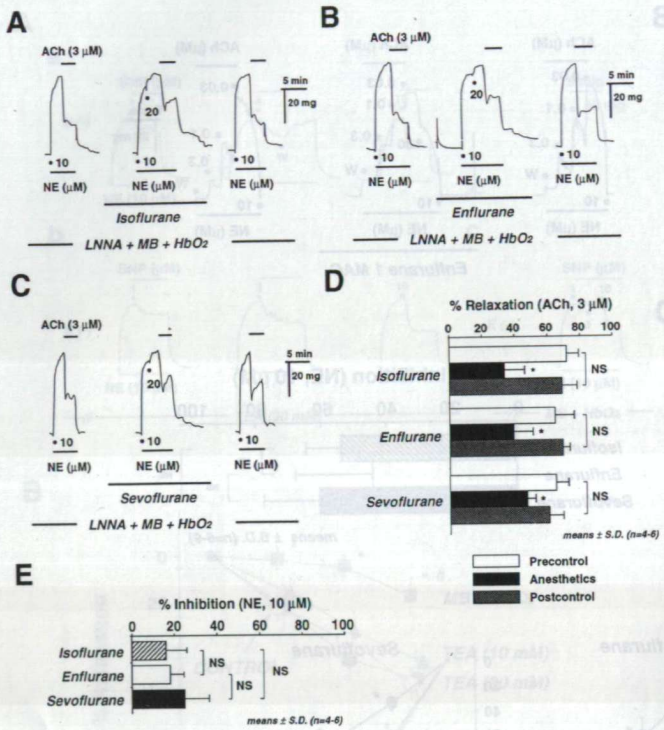

Fig. 9. (*A–C*) Examples of the inhibitory effects of isoflurane, enflurane, and sevoflurane on the acetylcholine (ACh, 3 μM)-induced relaxation in the endothelium-intact strips precontracted with norepinephrine (NE, 10 μM) in the presence of inhibitors of the EDRF pathway, including N^G-nitro L-arginine (LNNA, 100 μM), oxyhemoglobin (HbO_2, 10 μM), and methylene blue (MB, 10 μM). (*D*) The white, black, and hatched bars show the magnitude of the ACh relaxation before exposure to the anesthetics (precontrol), in the presence of the anesthetics, and after washout of the anesthetics from the bath (postcontrol), respectively. Values are mean ± SD (n = 4–6). No significant differences were observed in the ACh relaxation between the precontrol and postcontrol groups in all experiments. *$P < 0.05$ *versus* pre- and postcontrol. (*E*) Inhibitory effects of isoflurane, enflurane, and sevoflurane on the NE (10 μM)-induced contractions in the presence of LNNA, HbO_2, and MB. Values are mean ± SD (n = 4–6). NS = no significant differences between the groups.

meability, as previously described.[20,24,33] In low $[K^+]o$, the membrane potential deviated most from E_K and the ACh hyperpolarizations were the largest. Conversely, in the presence of high $[K^+]o$, where the membrane potential was essentially the same as E_K, ACh-induced hyperpolarizations were absent. In addition, the hyperpolarizations were almost completely inhibited in the presence of TEA (≥ 10 mM), a nonspecific K^+ channel blocker.[28,47,48] The lack of effect of glibenclamide (10 μM), a specific blocker of ATP-sensitive K^+ channels, and Ba^{2+} (100 μM) indicate that the ATP-sensitive or Ba^{2+}-sensitive K^+ channels are not involved in the generation of the hyperpolarization in the small rabbit mesenteric artery. Glibenclamide (10 μM) and Ba^{2+} (50 μM) have been shown to inhibit the ACh-induced hyperpolarization in rabbit cerebral arteries[33,49]; however, our results are similar to those recently reported in the guinea-pig coronary artery[36] and indicate there may be regional or species differences in the mechanisms behind the generation of ACh hyperpolarization. In addition, both 4AP-sensitive K^+ channels, *e.g.,* the low conductance (10 pS), Ca^{2+}-dependent, ATP-sensitive K^+ channel in portal vein,[50,51] and apamin-sensitive channels (0.1 and 0.3 μM), *i.e.,* the low conductance Ca^{2+}-activated K^+ channels,[28,52,53] appear to play little, if any, role in the ACh-induced hyperpolarization or relaxations, because 4AP and apamin had little, if any, effect on the responses to ACh. Finally, although we did not examine the effects of charybdotoxin, a specific inhibitor of high-conductance Ca^{2+}-activated K^+ channels (Big K^+[BK] channels),[28] the lack of significant effect of low concentrations (0.1–1 mM) of TEA on the hyperpolarization indicates that the BK channels do not play a significant role in the generation of the hyperpolarization. The BK channels are highly sensitive to TEA, with the reported EC_{50} values for block by external TEA being 100–300 μM, and BK currents are almost completely abolished by low concentrations of TEA.[28,54–57] In addition, we have previously reported that CTX (100 nM) did not affect the ACh-induced endothelium-dependent hyperpolarization in rabbit coronary artery,[27] and it is also known that CTX (100 nM) does not affect ACh (3 μM)- and bradykinin (0.1 μM)-induced endothelium-dependent hyperpolarization in rat mesenteric and canine coronary arteries, respectively (Drs. M. Nakashima and P. M. Vanhoutte, personal communication, 1994). Because nitric oxide has recently been shown to directly activate the BK channel in rabbit aortic smooth muscle,[58] the lack of involvement of BK channels is consistent with the proposal that the hyperpolarization is independent from the action of NO.

Although the observed depolarizing action of all the K^+ channel blockers with the exception of apamin implies that K^+ channels that are sensitive to these blockers may contribute to maintenance of the resting membrane potential, our observations indicate that the ACh hyperpolarization is mediated through the activation of TEA-sensitive K^+ channels that are distinct from those inhibited by glibenclamide (10 μM), Ba^{2+} (≤ 100 μM), 4AP (≤ 100 μM), or apamin (≤ 0.3 μM). However, a

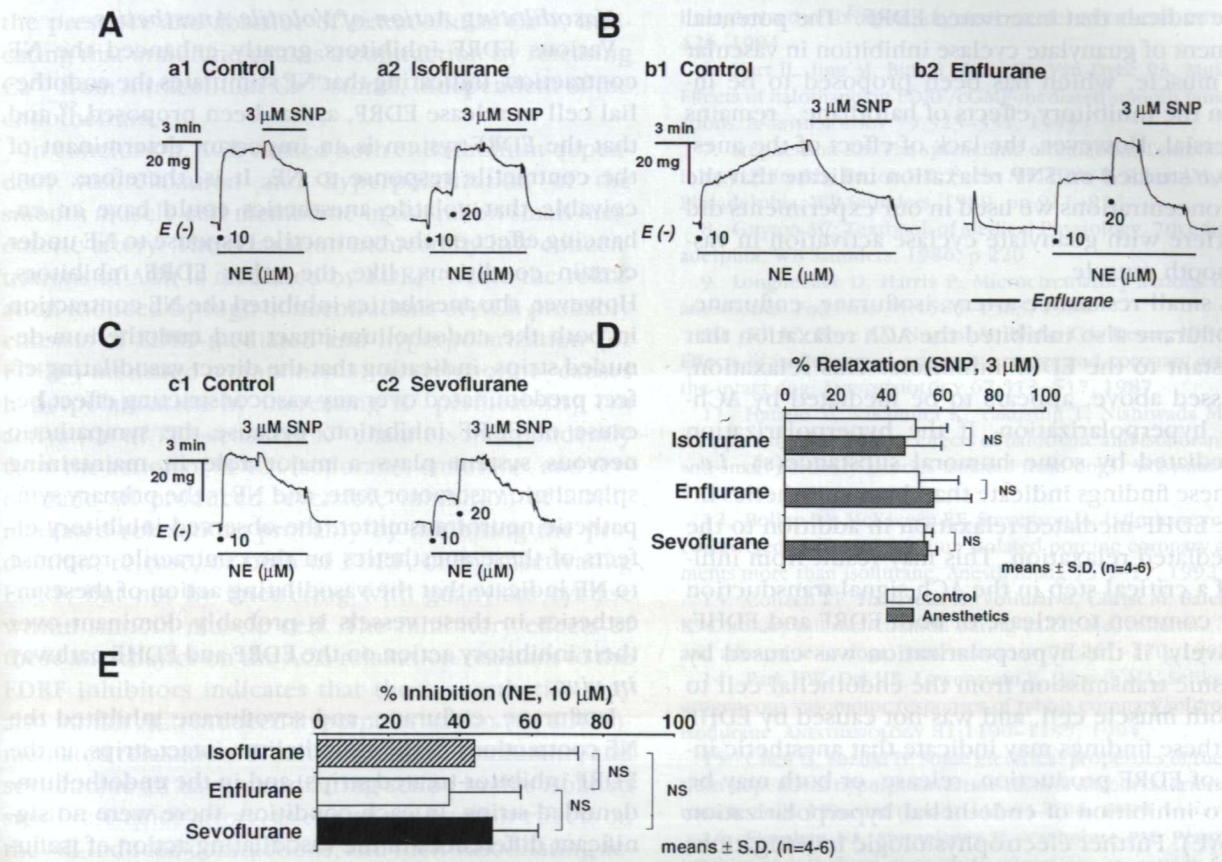

Fig. 10. (*A–C*) Examples of the effects of isoflurane, enflurane, and sevoflurane on the SNP-induced relaxation in the endothelium-denuded strips. (*D*) Effects of isoflurane, enflurane, and sevoflurane on the SNP-induced relaxation in the endothelium-denuded strips precontracted with norepinephrine (NE, 10 μM). The open and dark bars show the SNP relaxation before exposure to the anesthetics (control) and in the presence of the anesthetics, respectively. (*E*) Inhibitory effects of isoflurane, enflurane, and sevoflurane on the NE (10 μM)-induced contractions in the endothelium-denuded strips. Values are mean ± SD (n = 4–6). NS = no significant differences between the groups.

variety of K^+ channels are blocked by TEA, and the TEA sensitivity does not allow for a specific identification of the K^+ channels involved.[28,47,48] Further investigation will be necessary to clarify the specific channels, as well as the tissue type (*i.e.,* smooth muscle *versus* endothelium), that are responsible for this effect.

Effects of Volatile Anesthetics on the Acetylcholine Relaxation

Isoflurane, enflurane, and sevoflurane all caused a reversible inhibition of the relaxation induced by low concentrations of ACh (0.03–0.3 μM), which was highly sensitive to the EDRF (NO) inhibitors. This indicates that these volatile anesthetics inhibit the EDRF-mediated relaxation in this small splanchnic resistance artery, as observed in other large conductance arteries.[1–5] Furthermore, all three anesthetics had no effect on the SNP (3 μM ≈ IC_{50} value)-induced relaxation, which is presumably mediated through endothelium-independent NO generation from SNP in smooth muscle. Consistent with this, SNP-induced relaxation was inhibited by MB and HbO_2. These latter results indicate that the inhibition of the ACh-induced relaxation in these small mesenteric arteries was caused by inhibition of EDRF production, release from endothelium, or both, but not by an effect on vascular smooth muscle responsiveness to NO. This is consistent with previous observations regarding isoflurane and enflurane in rat aorta.[1,2,4,5] However, our data also may not be inconsistent with the mechanism proposed by Yoshida *et al.* for sevoflurane-induced inhibition of endothelium-dependent relaxation, which involved formation of ox-

ygen free radicals that inactivated EDRF.[3] The potential involvement of guanylate cyclase inhibition in vascular smooth muscle, which has been proposed to be involved in the inhibitory effects of halothane,[7] remains controversial. However, the lack of effect of the anesthetics we studied on SNP relaxation indicate that the 1 MAC concentrations we used in our experiments did not interfere with guanylate cyclase activation in vascular smooth muscle.

In this small resistance artery, isoflurane, enflurane, and sevoflurane also inhibited the ACh relaxation that was resistant to the EDRF inhibitors. This relaxation, as discussed above, appears to be mediated by ACh-induced hyperpolarization. If the hyperpolarization were mediated by some humoral substance(s), *i.e.,* EDHF, these findings indicate that these anesthetics inhibit the EDHF-mediated relaxation in addition to the EDRF-mediated relaxation. This may result from inhibition of a critical step in the ACh signal transduction pathway common to release of both EDRF and EDHF. Alternatively, if the hyperpolarization was caused by electrotonic transmission from the endothelial cell to the smooth muscle cell, and was not caused by EDHF release, these findings may indicate that anesthetic inhibition of EDRF production, release, or both may be related to inhibition of endothelial hyperpolarization (see above). Further electrophysiologic investigations regarding volatile anesthetic effects on the ACh-induced hyperpolarization of vascular smooth muscle cell membrane, as well as of vascular endothelial cell membrane, will be necessary to definitively solve these issues.

One possible explanation for the current findings is that volatile anesthetics may globally inhibit the two pharmacologically distinct ACh-induced vascular responses by interfering with muscarinic receptors. However, Muldoon *et al.* showed that halothane inhibits both ACh- and bradykinin-induced relaxations[1] and, more recently, Uggeri *et al.* showed that halothane, isoflurane, and enflurane inhibited not only receptor-mediated (methacholine-induced) relaxation but also nonreceptor-mediated (A23187-induced) endothelium-dependent relaxation.[2] Similarly, Yoshida has also shown that sevoflurane inhibits both bradykinin-induced and A23187-induced relaxation, as well as ACh-induced relaxation.[3] These results thus strongly indicate that the site of action is distal to the endothelial cell receptors. The observed volatile anesthetic effects on the ACh-mediated vascular responses are thus probably not caused by interference with muscarinic receptor activation.

Vasodilating Action of Volatile Anesthetics

Various EDRF inhibitors greatly enhanced the NE contraction, indicating that NE stimulates the endothelial cell to release EDRF, as has been proposed,[59] and that the EDRF system is an important determinant of the contractile response to NE. It is, therefore, conceivable that volatile anesthetics could have an enhancing effect on the contractile response to NE under certain conditions, like the other EDRF inhibitors. However, the anesthetics inhibited the NE contraction in both the endothelium-intact and endothelium-denuded strips, indicating that the direct vasodilating effect predominated over any vasoconstricting effect because of EDRF inhibition. Because the sympathetic nervous system plays a major role in maintaining splanchnic vasomotor tone, and NE is the primary sympathetic neurotransmitter, the observed inhibitory effects of these anesthetics on the contractile response to NE indicate that the vasodilating action of these anesthetics in these vessels is probably dominant over their inhibitory action on the EDRF and EDHF pathway *in vivo.*

Isoflurane, enflurane, and sevoflurane inhibited the NE contractions in the endothelium-intact strips, in the EDRF inhibitor-treated strips, and in the endothelium-denuded strips. In each condition, there were no significant differences in the vasodilating action of isoflurane, enflurane, and sevoflurane when compared at equi-MAC concentrations in rabbit. We did not compare the vasodilating action of each anesthetic between endothelium-intact and endothelium-denuded strips because all the inhibitors of the EDRF pathway significantly enhanced (1.7–2.4 times) the NE contraction in this artery, as previously observed.[17,26,27] This strongly indicates the possibility that the contractile response to NE would also be enhanced by the mechanical endothelial denudation. As previously discussed,[60,61] comparisons between the E(+) and E(−) strips are problematic when the contractile responses to NE are different and the vasodilating action of ACh is estimated according to the percentage decrement from the control response. However, we can conclude from our data that all these anesthetics have direct vasodilating actions on these isolated small splanchnic arteries, and that the vasodilating actions of all these anesthetics have a component that is independent of endothelium.

Finally, enflurane produced transient contractions in various conditions, *i.e.,* in the E(+) strips, in the EDRF inhibitor-treated strips and in the E(−) strips both in

the presence and absence of extracellular Ca^{2+}, indicating that enflurane causes a contraction by releasing Ca^{2+} from intracellular Ca^{2+} stores, independent of the endothelium.

In conclusion, ACh caused both endothelium-dependent vasorelaxation and hyperpolarization of the smooth muscle cell membrane in the rabbit small mesenteric artery. The relaxation induced by low concentrations of ACh is mediated by EDRF, while the relaxation induced by high concentrations of ACh probably consists of EDRF-mediated and hyperpolarization (or EDHF)-mediated components. Acetylcholine causes hyperpolarization by increasing K^+ permeability *via* activation of TEA-sensitive K^+ channels independently from the action of EDRF. Isoflurane, enflurane, and sevoflurane all produced reversible inhibition of EDRF-mediated relaxation, probably by inhibiting the production, release, or both of EDRF, or by inactivating EDRF, but not by interfering with guanylate cyclase within smooth muscle cell. The inhibitory effects of these anesthetics on the ACh relaxation resistant to the EDRF inhibitors indicates that these anesthetics may also inhibit ACh-induced hyperpolarization (or EDHF)-mediated relaxation. Finally, isoflurane, enflurane, and sevoflurane all have vasodilating action in this isolated small resistance artery, as evidenced by their effects on the NE-induced contractions, and their vasodilating action is, at least in part, endothelium independent.

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References

1. Muldoon SM, Hart JL, Bowen KA, Freas W: Attenuation of endothelium-mediated vasodilation by halothane. ANESTHESIOLOGY 68: 31–37, 1988

2. Uggeri MJ, Proctor GJ, Jones RA: Halothane, enflurane, and isoflurane attenuate both receptor- and non-receptor-mediated EDRF production in rat thoracic aorta. ANESTHESIOLOGY 76:1012–1017, 1992

3. Yoshida K, Okabe E: Selective impairment of endothelium-dependent relaxation by sevoflurane: Oxygen free radicals participation. ANESTHESIOLOGY 76:440–447, 1992

4. Toda H, Nakamura K, Hatano Y, Nishiwada M, Kakuyama M, Mori K: Halothane and isoflurane inhibit endothelium-dependent relaxation elicited by acetylcholine. Anesth Analg 75:198–203, 1992

5. Blaise G, To Q, Parent M, Lagarde B, Asenjo F, Sauve R: Does halothane interfere with the release, action, or stability of endothelium-derived relaxing factor/nitric oxide? ANESTHESIOLOGY 80:417–426, 1994

6. Hart JL, Jing M, Bina S, Freas W, Van Dyke RA, Muldoon SM: Effects of halothane on EDRF/cGMP-mediated smooth muscle relaxations. ANESTHESIOLOGY 79:323–331, 1993

7. Stephenson RB: The splanchnic circulation, Textbook of Physiology. Edited by Patton HD, Fuchs AH, Hille B, Scher AM, Steiner R. Philadelphia, WB Saunders, 1989, pp 911–923

8. Guyton AC: Textbook of Medical Physiology, 7th edition. Philadelphia, WB Saunders, 1986, p 220

9. Longnecker D, Harris P: Microcirculatory actions of general anesthetics. Fed Proc 39:1580–1583, 1980

10. Sill JC, Bove AA, Nugent M, Blaise GA, Dewey JD, Grabau C: Effects of isoflurane on coronary arteries and coronary arterioles in the intact dog. ANESTHESIOLOGY 67:513–517, 1987

11. Hatano Y, Nakamura K, Yakushiji T, Nishiwada M, Mori K: Comparison of the direct effects of halothane and isoflurane on large and small coronary arteries isolated from dogs. ANESTHESIOLOGY 73: 513–517, 1990

12. Bollen BA, McKlveen RE, Stevenson JA: Halothane relaxes preconstricted small and medium isolated porcine coronary artery segments more than isoflurane. Anesth Analg 75:9–17, 1992

13. Conzen PF, Habazetti H, Vollmar B, Christ M, Baier H, Peter K: Coronary microcirculation during halothane, enflurane, isoflurane and adenosine in dogs. ANESTHESIOLOGY 76:261–270, 1992

14. Park KW, Dai HB, Lowenstein E, Darvish MA, Sellke FW: Heterogeneous vasomotor responses of rabbit coronary microvessels to isoflurane. ANESTHESIOLOGY 81:1190–1197, 1994

15. Chen G, Suzuki H: Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. J Physiol (Lond) 410:91–106, 1989

16. Flavahan NA, Shimokawa H, Vanhoutte PM: Pertussis toxin inhibits endothelium-dependent relaxations to certain agonists in porcine coronary arteries. J Physiol (Lond) 408:549–560, 1989

17. Nishiye E, Nakao K, Itoh T, Kuriyama H: Factors inducing endothelium-dependent relaxation in the guinea-pig basilar artery as estimated from the actions of haemoglobin. Br J Pharmacol 96: 645–655, 1989

18. Rees DD, Palmer RMJ, Hodson HF, Moncada S: A specific inhibition of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation. Br J Pharmacol 96:418–424, 1989

19. Beny JL, Brunet PC: Neither nitric oxide nor nitroglycerin accounts for all the characteristics of endothelially mediated vasodilation of pig coronary arteries. Blood Vessels 25:308–311, 1988

20. Chen G, Suzuki H, Weston AH: Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. Br J Pharmacol 95:1165–1174, 1988

21. Feletou M, Vanhoutte PM: Endothelium-dependent hyperpolarization of canine coronary smooth muscle. Br J Pharmacol 93: 515–524, 1988

22. Taylor SG, Weston AH: Endothelium-derived hyperpolarizing factor: A new endogenous inhibitor from the vascular endothelium. Trends Pharmacol Sci 9:272–274, 1988

23. Suzuki H, Yamamoto Y: Endothelium-derived hyperpolarizing factor (EDHF)—A vasodilating substance by activating K-channels, Ion Channels of Vascular Smooth Muscle Cells and Endothelial Cells. Edited by Sperelakis N, Kuriyama H. New York, Elsevier Science Publishing Co., Inc., 1991, pp 173–184

24. Komori K, Lorenz RR, Vanhoutte PM: Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. Am J Physiol 255:H207–H212, 1988

25. Garland CJ, Mcpherson GA: Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. Br J Pharmacol 105:429–435, 1992

26. Akata T, Yoshitake J, Nakashima M, Itoh T: Effects of protamine on vascular smooth muscle of rabbit mesenteric artery. ANESTHESIOLOGY 75:833–846, 1991

27. Nakashima M, Akata T, Kuriyama H: Effects on the rabbit coronary artery of LP-805, a new type of releaser of endothelium-derived relaxing factor and a K^+ channel opener. Circ Res 71:859–869, 1992

28. Cook NS: The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol Sci 9:21–28, 1988

29. Drummond JC: MAC for halothane, enflurane, and isoflurane in the New Zealand white rabbit: And a test for the validity of MAC determinations. ANESTHESIOLOGY 62:336–338, 1985

30. Scheller MS, Saidman LJ, Partridge BL: MAC of sevoflurane in humans and the New Zealand white rabbit. Can J Anaesth 35:153–156, 1988

31. Ignarro LJ, Gold ME, Buga GM, Byrns RE, Wood KS, Chaudhuri G, Frank G: Basic polyamino acids rich in arginine, lysine, ornithine cause both enhancement of and refractoriness to formation of endothelium-derived nitric oxide in pulmonary artery and vein. Circ Res 64:315–29, 1989

32. F. Renzi BA, Waud BE: Partition coefficients of volatile anesthetics in Krebs solution. ANESTHESIOLOGY 47:62–63, 1977

33. Brayden JE: Membrane hyperpolarization is a mechanism of endothelium-dependent cerebral vasodilation. Am J Physiol 259: H668–H673, 1990

34. Tare M, Parkington HC, Coleman HA, Neild TO, Dusting GJ: Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. Nature 346:69–71, 1990

35. Ito S, Kajikuri J, Itoh T, Kuriyama H: Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity by noradrenaline in the rabbit mesenteric artery. Br J Pharmacol 104:227–233, 1991

36. Chen G, Yamamoto Y, Miwa K, Suzuki H: Hyperpolarization of arterial smooth muscle induced by endothelial humoral substances. Am J Physiol 260:H1888–H1892, 1991

37. Busse R, Fichtner H, Lückhoff A, Kohlhardt M: Hyperpolarization and increased free calcium in acetylcholine-stimulated endothelial cells. Am J Physiol 255:H965–H969, 1988

38. Davies PF, Olesen SP, Clapham DE, Morrel EM, Schoen FJ: Endothelial communication. Hypertension 11:563–572, 1988

39. Groschner K, Graier WF, Kukovetz WR: Activation of a small-conductance Ca^{2+}-dependent K^+ channel contributes to bradykinin-induced stimulation of nitric oxide synthesis in pig aortic endothelial cells. Biochem Biophys Acta 1137:162–170, 1992

40. Nilius B: Ion channels and regulation of transmembrane Ca^{2+} influx in endothelium, Ion Channels of Vascular Smooth Muscle Cells and Endothelial Cells. Edited by Sperelakis N, Kuriyama H. New York, Elsevier Science Publishing Co., Inc., 1991, pp 317–325

41. Lückhoff A, Busse R: Calcium influx into endothelial cells and formation of endothelium-derived relaxing factor is controlled by the membrane potential. Pflügers Arch 416:305–311, 1990

42. Rhodin JAG: The ultrastructure of mammalian arterioles and precapillary sphincters. J Ultrastructure Res 18:181–223, 1967

43. Spagnoli LG, Villaschi S, Neli L, Palmieri G: Gap junctions in myo-endothelial bridges of rabbit carotid arteries. Experientia 38: 124–125, 1982

44. Kauser K, William WJ, Rubanyi G, Harder DR: Mechanisms of action of EDRF on pressurized arteries: Effect on K^+ conductance. Circ Res 65:199–204, 1989

45. Beny JL: Endothelial and smooth muscle cells hyperpolarized by bradykinin are not dye coupled. Am J Physiol 258:H836–H841, 1990

46. Mülsch A, Busse R: N^G-nitro-L-arginine (N5-[imino(nitro-amino)methyl]-L-ornithine) impairs endothelium-dependent dilations by inhibiting cytosolic nitric oxide synthesis from L-arginine. Naunyn Schmiedebergs Arch Pharmacol 341:143–147, 1990

47. Langton PD, Nelson MT, Huang Y, Standen NB: Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 260:H927–H934, 1991

48. Stanfield PR: Tetraethylammonium ions and the potassium permeability of excitable cells. Rev Physiol Biochem Pharmacol 97: 1–67, 1983

49. Standen NB, Quayle JM, Davies NW, Braydon JE, Huang Y, Nelson MT: Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. Science 245:177–180, 1989

50. Okabe K, Kitamura K, Kuriyama H: Features of 4-aminopyridine sensitive outward current observed in single smooth muscle cells from the rabbit pulmonary artery. Pflügers Arch 409:561–568, 1987

51. Kajioka S, Oike M, Kitamura K: Nicorandil opens a calcium-dependent potassium channel in smooth muscle cells of the rat portal vein. J Pharmacol Exp Ther 254:905–913, 1990

52. Banks BEC, Brown C, Brugess GM, Burnstock G, Claret M, Cooks TM, Jenkinson DH: Apamin blocks certain neurotransmitter-induced increases in potassium permeability. Nature 282:415–417, 1979

53. Romey G, Hughes M, Schmid-Antomarchi H, Lazdunski M: Apamin: A specific toxin to study a class of Ca^{2+}-dependent K^+ channels. J Physiol (Paris) 79:259–264, 1984

54. Langton PD, Nelson MT, Huang Y, Standen NB: Block of calcium-activated potassium channels in mammalian arterial myocytes by tetraethylammonium ions. Am J Physiol 260:H927–H934, 1991

55. Latorre R, Vergara C, Hidalgo C: Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. Proc Natl Acad Sci U S A 79:804–809, 1982

56. Blatz AL, Magleby KL: Ion conductance and selectivity of single calcium-activated potassium channels in cultured rat muscle. J Gen Physiol 84:1–23, 1984

57. Nelson MT, Brayden JE: Regulation of arterial tone by calcium-dependent K^+ channels and ATP-sensitive K^+ channels. Cardiovasc Drugs Ther 7:605–610, 1993

58. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA: Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 368:850–853, 1994

59. Luscher TF, Vanhoutte PM: The Endothelium: Modulator of Cardiovascular Function. Boca Raton, CRC press, 1990, pp 1–6

60. Akata T, Kodama K, Yoshitake J, Takahashi S: Heparin prevents the vasodilating actions of protamine on human small mesenteric arteries. Anesth Analg 76:1213–1221, 1993

61. Akata T, Kodama K, Takahashi S: Effects of heparin on the vasodilator action of protamine in the rabbit mesenteric artery. Br J Pharmacol 109:1247–1253, 1993