Anesthesiology
77:482–487, 1992

Role of Guanylate Cyclase–cGMP Systems in Halothane-induced Vasodilation in Canine Cerebral Arteries

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The cellular mechanisms through which halothane dilates blood vessels remain largely unknown. The present studies were designed to determine the effects of 0.59 and 0.9 mM halothane (equivalent to 2.0% and 3.0%, respectively) on tissue cyclic guanosine 3,5-monophosphate (cGMP) level and guanylate cyclase enzyme activity in canine middle cerebral arteries. Rings of cerebral arteries preconstricted with 5-hydroxytryptamine (0.2 μM) were exposed for 15 min to low or high concentrations of halothane or for 5 min to sodium nitroprusside (50 μM). The vessels were instantaneously frozen by immersing them in liquid N_2; they then were homogenized, and the tissue cGMP levels were determined using radioimmunoassay. Halothane produced 2.23 ± 0.44- and 4.47 ± 0.87-fold increases in tissue cGMP levels over control at 0.59 and 0.9 mM, respectively. Sodium nitroprusside, a nitrovasodilator, also increased the tissue cGMP level 7.80 ± 1.36-fold over the control value. To understand better the mechanisms of halothane-induced increase of tissue cGMP level, the effects of this anesthetic agent on guanylate cyclase enzyme activity were examined. Halothane, unlike sodium nitroprusside, did not modulate the activity of the soluble guanylate cyclase enzyme. However, halothane (1.0 mM), like atrial natriuretic factor (5 μM), stimulated the particulate guanylate cyclase enzyme activity. LY-83583 (6-anilino-5,8-quinolinedione, 10 μM), an agent that inhibits soluble guanylate cyclase activity, significantly reduced the response of the vessels to calcium ionophore (A23187, 0.4 μM), an endothelium-dependent vasodilator, without producing a significant effect on halothane-induced vasodilation. These results suggest that halothane-induced vasodilation of cerebral blood vessels is partly mediated by an increase in tissue cGMP levels. The increase in cGMP level induced by halothane results, at least in part, from activation of the particulate but not the soluble guanylate cyclase enzyme in canine middle cerebral vessels. The present study, however, does not rule out the possible interaction of halothane with the cGMP phosphodiesterase enzymes. (Key words: Anesthetics, volatile: halothane. Circulation, cerebral: middle cerebral artery. Enzyme, guanylate cyclase: particulate; soluble. Hormones: atrial natriuretic factor. LY-83583. Nucleotide: cyclic guanosine 3,5-monophosphate.)

CYCLIC GUANOSINE 3,5-monophosphate (cGMP) plays an important role in modulating vascular smooth muscle tone. Nitrovasodilators, endothelium-derived relaxing factor, and atrial natriuretic factor all induce vasorelaxation and increase tissue cGMP level by enhancing guanylate cyclase activity.[1–3] Direct application of the 8-bromo derivative of cGMP relaxes preconstricted vascular smooth muscle[4] and mimics the vasodilator action of nitroglycerin.[5] In most tissues, there are two guanylate cyclase isoenzymes, a membrane-bound particulate and a cytosolic soluble enzyme. The effects of nitrovasodilators and the endothelium-dependent vasodilators is thought to be mediated by nitric oxide radicals that directly activate the soluble guanylate cyclase enzyme.[6] In the case of atrial natriuretic factor, production of cGMP is mediated by their binding to cell surface receptors, which then activate the membrane-bound particulate guanylate cyclase.[3] The increase in cGMP level subsequently leads to vasodilation.

Halothane is a potent cerebral vasodilator, increasing cerebral blood flow in a concentration-dependent manner both in humans[7] and animals.[8] Although halothane is a potent vasodilator, the biochemical mechanisms by which halothane induces vasodilation are not well understood. Sprague *et al.*[9] found that halothane and isoflurane increase intracellular cyclic 3,5-adenosine monophosphate (cAMP) in rat aortic smooth muscle. Halothane has been reported to increase cGMP levels both in mouse myocardium[10] and cortex[11] but to decrease cGMP level in the cerebellum.[11] However, the direct effect of this volatile anesthetic on vascular smooth muscle tissue cGMP levels and guanylate cyclase enzyme activity has not been reported. Therefore, the purposes of the present study were 1) to determine the effects of halothane on tissue cGMP level; 2) to determine the direct effects of halothane on soluble and particulate guanylate cyclase enzyme activities; and 3) to examine if the relaxation in canine middle cerebral arteries produced by halothane is blocked by LY-83583, an inhibitor of soluble guanylate cyclase.

Materials and Methods

These experiments were approved by the Medical College of Wisconsin Animal Care Committee.

VESSEL ISOLATION

Adult mongrel dogs weighing 15–25 kg were killed during anesthesia (sodium thiamylal 30 mg/kg intrave-

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Received from the Departments of Anesthesiology, Pharmacology and Physiology, The Medical College of Wisconsin, Milwaukee, Wisconsin. Accepted for publication April 14, 1992. Supported in part by National Institutes of Health grant HL 01901 and Anesthesiology Research Training grant GM 08377.

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nously), and their brains were removed. The middle cerebral arteries were carefully dissected and cleaned of adherent connective tissues. Both isometric contractions and cGMP content were determined in endothelium-intact middle cerebral arterial rings that were incubated in physiologic salt solution (*p*H 7.4) of the following composition (millimolar): NaCl 119, KCl 4.7, $CaCl_2$, 1.6, $MgSO_4$ 1.17, $NaHCO_3$ 27.8, NaH_2PO_4 1.18, EDTA 0.026, glucose 5.5, and HEPES 5 [4-(2-hydroxyethyl) -1-piperazineethane-sulfonic acid]. The solution was aerated with 93.5% O_2 and 6.5% CO_2.

Isometric Tension Recording

Rings of middle cerebral arteries were mounted between two tungsten wire triangles in 15-ml water-jacketed organ baths. Isometric tension was recorded on a Grass polygraph (model 7). The temperature was maintained at 37° C. The rings were progressively stretched to a final optimal tension of approximately 750 mg. The optimal tension was determined previously by length–tension studies using 40 mM KCl.** An equilibration period of 90 min was allowed before the start of the experiment. The integrity of each ring was examined by the contractile response to 40 mM KCl added in the bathing media. The rings were washed repeatedly, and after equilibration the rings were contracted with 5-hydroxytryptamine (5-HT, 0.2 μM) in the absence and presence of LY-83583 (10 μM). At least 60 min elapsed between successive exposures to 5-HT. At a stable plateau tension, different concentrations of halothane were bubbled for 10–15 min into the tissue bath using a vaporizer (Dragerwerk, Lubeck, Germany) in a randomized fashion to determine dose–response relations. The concentration of halothane in the bath was determined by gas chromatography, and its vapor content in the carrier gases was measured by mass spectrometry.

Assay of Cyclic Nucleotides

Unstretched vessels were precontracted with 0.2 μM 5-HT and exposed to halothane (0.59 or 0.9 mM) for 15 min or to 50 μM sodium nitroprusside for 5 min. This time of exposure was chosen because the relaxation induced by halothane was maximal and stable after 10–15 min exposure. Two vessels were used as parallel control, constricted with 5-HT but not exposed to halothane or sodium nitroprusside. The vessels were then immediately immersed in liquid N_2. The preparations were homogenized in 6% trichloroacetic acid and centrifuged at 3,000 × g for 15 min. Trichloroacetic acid was extracted from the supernatant with water-saturated ether. cGMP content was then measured in aliquots of the supernatant by radioimmunoassay (Ammersham). Protein concentrations were determined with the method of Lowry *et al.*[12]

Guanylate Cyclase Assay

Arteries were homogenized in cold 0.25 M sucrose containing 5 mM Tris-HCl (*p*H 7.4). A crude soluble extract was obtained by centrifugation at 37,000 × g for 60 min at 4° C. The supernatant was recovered and used in the soluble guanylate cyclase assay. The pellet was resuspended in the homogenization buffer and used in the particulate guanylate cyclase determinations, essentially as described by Winquist *et al.*[3]

The guanylate cyclase assay was performed in an assay medium of the following composition: 50 mM Tris-HCl (*p*H = 7.4), 10 mM theophylline, 15 mM creatine phosphate, creatine phosphokinase (135 units/mg protein), 2.5 mM isobutylmethylxanthine, 5 mM guanosine triphosphate (GTP), 4 mM $MgCl_2$, and 30–70 μg membrane protein in a final volume of 100 μl. Reactions were initiated by adding substrate (4 mM $MgCl_2$, 5 mM GTP) and terminated 15 min later by adding 150 μl cold Tris-HCl (50 mM) and heating for 3 min at 85° C. cGMP levels were quantified by radioimmunoassay. Liquid halothane was equilibrated in the assay buffer, and 30 μl of this solution was injected into the assay tubes immediately before the addition of the substrate to the media. Halothane concentration was verified by gas chromatography.

Data Analysis

Results are expressed as mean ± SEM (n = number of experiments, n ≥ 4). A Student's *t* test for unpaired or paired observations was used to evaluate statistical significance. A significant level of difference was considered at $P < 0.05$. The millimolar concentrations of halothane measured in the bath were converted to equivalent partial pressures in the solution and expressed as percentages of the volatile agent in the gas phase.[13]

Results

Effect of Halothane on Tissue cGMP Level

Addition of halothane (0.59 and 0.9 mM) produced a concentration-dependent increase in tissue cGMP level in rings precontracted with 0.2 μM 5-HT (fig. 1). The basal levels of cGMP in control vessels were 1.0 ± 0.2 pmol/mg protein. Fifteen minutes' exposure of the vessels to halothane at 0.59 and 0.9 mM increased tissue cGMP levels to 2.3 ± 0.6 and 4.6 ± 0.9 pmol/mg protein, respectively. After exposure to 50 μM of sodium nitroprusside for 5 min, the cGMP level was increased to 7.8 ± 1.4 pmol/mg protein.

** Flynn W: Unpublished observation.

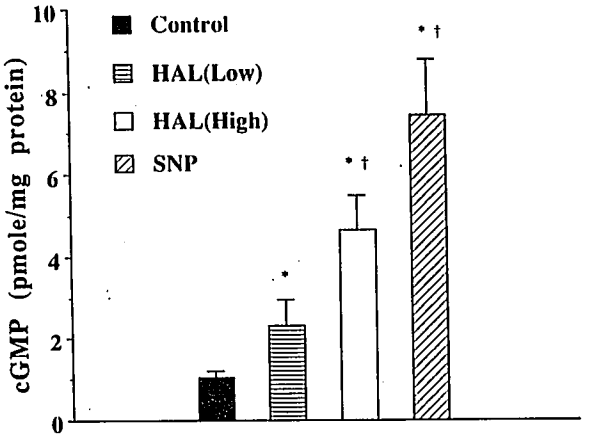

FIG. 1. Effects of low (0.59 mM, equivalent to 2.0%, n = 6) and high (0.9 mM, equivalent to 3.0%, n = 6) concentrations of halothane (HAL) and sodium nitroprusside (SNP, 50 μM, n = 6) on tissue cGMP levels of canine middle cerebral arteries that were precontracted with 0.2 μM 5-HT. Halothane increased tissue cGMP levels in a concentration-dependent manner. *Significantly different from the control value ($P < 0.05$). †Significantly different from the low halothane value ($P < 0.05$).

EFFECT OF HALOTHANE ON SOLUBLE AND PARTICULATE GUANYLATE CYCLASE ENZYME ACTIVITY

To determine and compare the effects of halothane and sodium nitroprusside on soluble guanylate cyclase activity, the soluble form of the enzyme was obtained from homogenates of middle cerebral arteries. The activity of the soluble enzyme in the presence of 50 μM sodium nitroprusside was significantly higher (30.8 ± 4.4 pmol/100 mg protein) than in control incubations (1.5 ± 0.3 pmol/100 mg protein; fig. 2). Halothane (1 mM, equivalent to 3.3%) had no effect on the enzymatic activity of the soluble guanylate cyclase.

The effects of halothane (1 mM) and atrial natriuretic factor (50 μM) on particulate guanylate cyclase activity prepared from the membrane fractions of canine middle cerebral arteries were also measured. The enzymatic activity in the presence of halothane was significantly higher (4.8 ± 1.4 pmol/mg protein) than in control incubations (1.6 ± 0.4 pmol/mg protein; fig. 3). Atrial natriuretic factor (50 μM) also significantly increased (6.4 ± 2.03 pmol/mg protein) the particulate enzyme activity as compared to the control. Guanylate cyclase activity increased in a linear fashion at incubation times up to 30 min (fig. 4). The presence of halothane produced an increase in particulate guanylate cyclase activity at each incubation time and did not alter the time course.

EFFECT OF LY-83583 ON HALOTHANE-INDUCED VASODILATION

Because halothane had no direct effect on soluble guanylate cyclase enzyme activity, we examined the effect of LY-83583, an inhibitor of soluble guanylate cyclase enzyme, on halothane-induced vasodilation in canine middle cerebral arteries. Typical recordings of halothane-induced relaxations in isolated middle cerebral arterial rings precontracted with 0.2 μM 5-HT are shown in figures 5A and 5B. Halothane produced concentration-dependent vasodilation (fig. 5C), and pretreatment of middle cerebral arteries with LY-83583 (10 μM) for 15 min produced no significant effect on halothane-induced vasodilation. However, the same concentration of LY-83583 produced 86 ± 6% inhibition of the response of the vessels

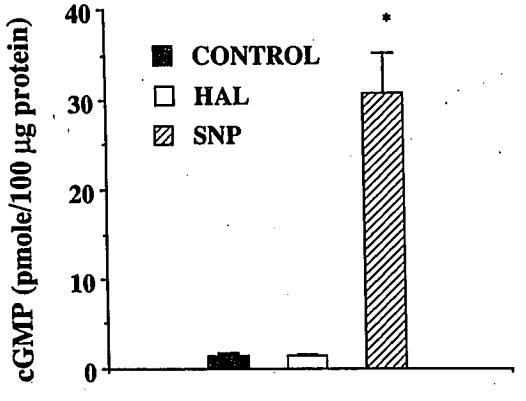

FIG. 2. Effects of halothane (HAL, 1.0 mM, equivalent to 3.3%, n = 4) and sodium nitroprusside (SNP, 50 μM, n = 4) on soluble guanylate cyclase activity from the soluble fractions of canine middle cerebral arteries. Basal soluble guanylate cyclase activity was 1.5 ± 0.3 pmol/100 mg protein. The activity of the enzyme in the presence of halothane was not significantly different than in control incubations. However, in the presence of SNP, the activity was increased to 30.8 ± 4.4 pmol/100 mg protein. *$P < 0.05$ *versus* control.

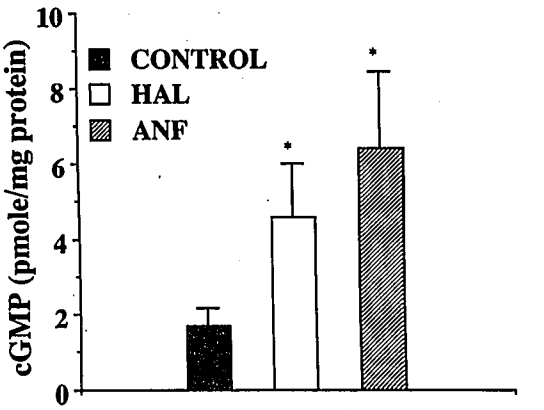

FIG. 3. Effects of halothane (HAL, 1.0 mM, equivalent to 3.3%, n = 7) and atrial natriuretic factor (ANF, 50 μM, n = 7) on particulate guanylate cyclase activity from the membrane fractions of canine middle cerebral arteries. Basal particulate guanylate cyclase activity = 1.6 ± 0.4 pmol/mg protein. The activities of the enzyme in the presence of halothane or ANF were significantly greater than that in control incubations. *Significantly different from the control value ($P < 0.05$).

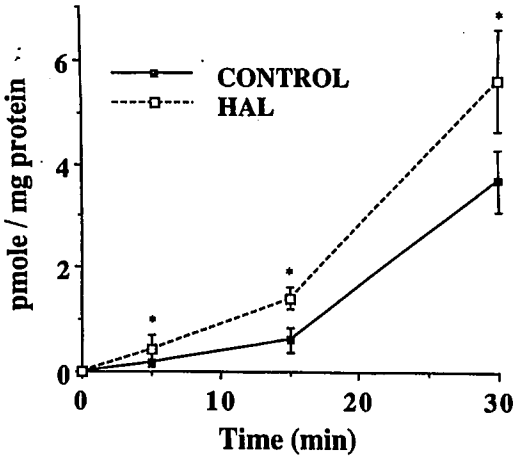

FIG. 4. Accumulation of cGMP in the incubation medium as a function of time. The membrane fractions of the homogenates were incubated in the absence (n = 3) and presence of halothane (HAL, 1.0 mM, equivalent to 3.3%, n = 2) for the indicated time. *Significantly different from the control value ($P < 0.05$).

to calcium ionophore (A23187, 0.4 μM), an endothelium-dependent vasodilator that works through the soluble isoform of guanylate cyclase.

Discussion

Previous studies have demonstrated the vasodilator effects of volatile anesthetics on isolated rabbit,[14] cat,[15] and dog[16] cerebral arteries. However, little is known about the subcellular mechanisms of volatile anesthetic-induced vasodilation. In the present study, halothane produced a concentration-dependent increase of tissue cGMP in 5-HT–precontracted canine middle cerebral arteries. These results suggest that the vasodilator action of halothane is mediated in part by increased vascular smooth muscle cGMP. It is now well established that an increase in cellular cGMP leads to vascular smooth muscle relaxation.[1-6] The present findings are consistent with the results obtained by Vulliemoz *et al.*[10] on mouse myocardium and cerebral cortex.[11] The increase in cardiac cGMP levels by halothane has been reported to involve postsynaptic α-adrenergic pathways.[10] However, because there are very few adrenergic receptors in cerebral blood vessels,[17] it appears that, unlike in mice ventricular myocardium, halothane-induced cGMP elevation in cerebral blood vessels may involve nonadrenergic pathways.

Guanylate cyclase, an enzyme that catalyzes the formation of cGMP from GTP, is associated with both the soluble and particulate (membrane) fractions of homogenates from a variety of tissues. The relative amounts of each of these forms vary with cell type and the protocol used for enzyme assay.[6] To determine if halothane interacts directly with guanylate cyclase enzyme, soluble and membrane fractions were prepared from canine middle cerebral arteries. Halothane produced no significant effect on the activity of the soluble guanylate cyclase enzyme. Furthermore, the soluble guanylate cyclase inhibitor, LY-83583,[18] did not alter halothane-induced vasodilation. Therefore, activation of the soluble guanylate cyclase is not involved in the halothane-induced increase in cGMP levels in canine cerebral arteries at the concentrations studied.

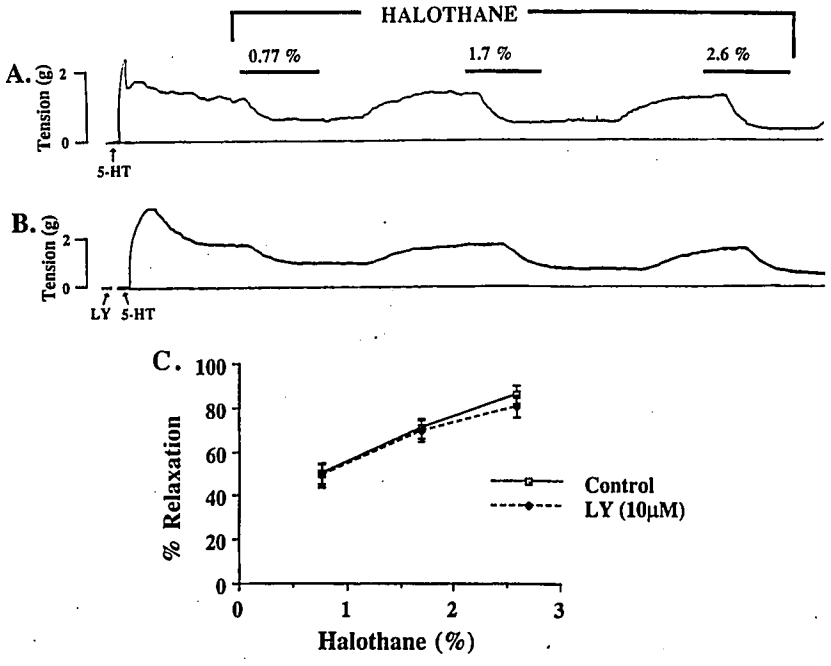

FIG. 5. Effect of LY-85385 (10 μM) on halothane-induced relaxation in middle cerebral arteries. *A, B:* Representative isometric tension recording of the effect of halothane on 5-hydroxytryptamine (5-HT, 0.2 μM)–precontracted artery before and after 15 min exposure to LY-85385, respectively. *C:* concentration–response curves of halothane before and after exposure to LY-85385. Data are expressed as percent relaxations of the 5-HT–induced contraction (n = 5).

However, the same concentration of halothane that produced no significant effect on the soluble guanylate cyclase stimulated particulate guanylate cyclase. The exact mechanism by which halothane increases particulate guanylate cyclase activity is beyond the scope of the present study. However, Lad[19,20] has suggested that an increase in membrane fluidity or membrane disorganization may be the mechanism by which agents such as filipin, digitonin, cereolysin, and streptolysin activate rat lung particulate guanylate cyclase. Even though halothane, like most volatile anesthetics, increases membrane fluidity,[21] further investigation is needed if such a mechanism also may explain the activation of particulate guanylate cyclase by halothane. In our experiments, a higher concentration of halothane was required to increase particulate guanylate cyclase activity than was needed to increase tissue cGMP levels. A reason for this discrepancy might be that the guanylate cyclase assay may be too insensitive to small changes in enzyme activity or that halothane at lower doses might not activate this enzyme. In the present study, 2.5 mM isobutylmethylxanthine and 10 mM theophylline were used in the assay medium to block the hydrolysis of cGMP by phosphodiesterases, and therefore a possible additional interaction of halothane with cGMP phosphodiesterase enzymes cannot be excluded.

cGMP induces vascular smooth muscle relaxation by activating cGMP-dependent protein kinase and altering the phosphorylation state of intracellular proteins, including the dephosphorylation of myosin light chain,[22,23] as well as by lowering intracellular Ca^{2+} level through activation of sarcolemmal Ca^{2+} ATPase.[24] At the present time there is no evidence showing the effect of volatile anesthetics on phosphorylation of proteins or dephosphorylation of myosin light chain in intact vascular smooth muscle. Halothane has been reported to impair the activation of isoproterenol-stimulated lipolysis in isolated rat adipocytes by cAMP-dependent protein kinase.[25] However, further investigation is necessary to examine the effect of halothane on cGMP-dependent protein kinases.

The present study has demonstrated that there is an association of the vasodilator action of halothane with an increase in cGMP levels. Volatile anesthetics have been reported to have nonselective effects on voltage-gated membrane K^+,[26] Na^+,[27] and Ca^{2+} channels in myocardial membranes.[28] Halothane also was observed to depress K^+ channel activity in vascular smooth muscle cells.†† The nonselective effects of volatile anesthetics on voltage-gated membrane ion channels might be a direct membrane effect. However, there is increasing evidence that cGMP modulates ion channels. For example, the soluble analogue of cGMP, 8-bromo derivative of cGMP, inhibited the inwardly rectifying K^+ channels of cultured ventricular myocytes from embryonic chick heart.[29] Intracellular perfusion of isolated cardiac myocytes with cGMP also reduces the amplitude of sarcolemmal Ca^{2+} current activated by cAMP-dependent mechanisms.[30] Therefore, even though halothane can directly modulate ion channels, some of its effects on ion channels may be mediated by an increase in tissue cGMP.

Several studies have demonstrated agonist-induced vasoconstrictor responses to be more susceptible than high K^+-induced responses to relaxation by volatile anesthetics.[15,31] On the other hand, Ca^{2+}-channel blocking agents, to which anesthetics have frequently been compared, are more efficacious in relaxing K^+-induced constriction than agonist-induced responses.[32,33] K^+-evoked contraction occurs as a result of depolarization followed by activation of voltage-gated Ca^{2+} channels, whereas agonist-induced contractions result from both extracellular Ca^{2+} influx and intracellular Ca^{2+} mobilization.[34] These results indicate the mechanism of halothane-induced vasodilation to be different from that of Ca^{2+} channel blocking agents. However, nitrovasodilators[35] and atrial natriuretic factor[36] have been shown to preferentially suppress agonist-induced responses, thus resembling halothane-induced vasodilation. Taken together, these previous observations in conjunction with the present study suggest a common mode of action for relaxation of vascular tissue by these agents and by halothane.

In conclusion, halothane produced concentration-dependent vasodilation and an increase in tissue cGMP in canine middle cerebral arteries. The increase in cGMP induced by halothane results, at least in part, from activation of the particulate but not the soluble guanylate cyclase enzyme.

The authors thank Eli Lilly & Company for their generous supply of LY-83583. They also thank Ms. Mimi Mick and Ms. Suzanne Emmrich for their excellent secretarial assistance and Ms. Mary Zibell for her excellent technical assistance.

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