Anesthesiology
74:97–104, 1991

The Influence of Inhalational Anesthetics on In Vivo *and* In Vitro *Benzodiazepine Receptor Binding in the Rat Cerebral Cortex*

Thomas D. Hansen, M.D.,* David S. Warner, M.D.,† Michael M. Todd, M.D.,‡
Max T. Baker, Ph.D.,§ Niels F. Jensen, M.D.¶

The effect of volatile anesthetics on benzodiazepine receptor binding was examined autoradiographically in the rat brain both *in vivo* and *in vitro* with the use of [^{3}H]-Ro-15-1788, a benzodiazepine antagonist. For *in vitro* studies, slide-mounted brain sections were incubated at 37° C in Tris buffer (50 mM, *p*H 7.4) with [^{3}H]-Ro-15-1788 (flumazenil, 0.5–12.0 nM) in the presence of air (control) or 1 MAC concentrations of halothane or isoflurane. Brain sections were exposed to x-ray film and their images digitized, and specific cortical [^{3}H]-Ro-15-1788 binding was determined. A Scatchard plot of specific cortical binding was constructed, and the dissociation constant (K_D) and maximum bound ligand per milligram tissue (B_{max}) were determined for each experimental group. In the *in vivo* trials, rats were anesthetized with 1 MAC halothane or isoflurane; 0.5 μCi/g [^{3}H]-Ro-15-1788 was given intravenously, and the animals were killed 15 min later. Seven standardized sagittal brain sections were examined from autoradiographs. Mean specific cortical binding was determined for each group and was compared with binding in unanesthetized control rats. A third experimental trial analyzed the timed arterial blood history of [^{3}H]-Ro-15-1788 in animals prepared exactly as in the *in vivo* study. The [^{3}H]-Ro-15-1788 blood clearance over 20 min and plasma [^{3}H]-Ro-15-1788 levels at 15 min after injection of isotope were evaluated. *In vitro* Scatchard analysis showed no difference in experimental groups in K_D or B_{max} at 37° C. *In vivo* trials demonstrated enhanced cortical binding in animals treated with halothane or isoflurane (halothane = 125 ± 23 fmol/mg; isoflurane = 130 ± 25 fmol/mg) compared with control animals (80 ± 9 fmol/mg). However, plasma levels at 15 min were higher in anesthetic-treated groups (halothane = $8.13 \pm 0.86 \cdot 10^{-7}$ mg/ml; isoflurane = $7.91 \pm 1.0 \cdot 10^{-7}$) compared with controls ($6.14 \pm 0.53 \cdot 10^{-7}$ mg/ml). Furthermore, the clearance of [^{3}H]-Ro-15-1788 was reduced in the presence of anesthetics (halothane = 6.46 ± 0.40 $ml \cdot kg^{-1} \cdot min^{-1}$; isoflurane = 6.42 ± 0.61 $ml \cdot kg^{-1} \cdot min^{-1}$) compared with in unanesthetized animals (7.64 ± 0.53 $ml \cdot kg^{-1} \cdot min^{-1}$). These findings indicate that neither halothane nor isoflurane acts directly through the benzodiazepine receptor *in vitro*. *In vivo*, volatile anesthetics apparently enhance cortical receptor binding, however, this difference can be explained in part by factors not directly related to receptor–anesthetic interactions, *i.e.*, reduced blood ligand clearance during anesthesia. (Key words: Anesthetics, volatile: halothane; isoflurane. Antagonists, benzodiazepines: Ro-15-1788 (flumazenil). Measurement techniques: autoradiography. Receptor binding.)

* Resident, Department of Anesthesiology.
† Associate Professor, Department of Anesthesiology.
‡ Professor, Department of Anesthesiology.
§ Assistant Professor, Department of Anesthesiology.
¶ Associate, Department of Anesthesiology, University of Iowa.

Received from the Neuroanesthesia Research Group, Department of Anesthesia, The University of Iowa College of Medicine, Iowa City, Iowa. Accepted for publication August 14, 1990. Supported in part by National Institutes of Health grant R29 GM39771 to Dr. Warner and R01 NS 24517 to Dr. Todd. Dr. Warner is a Parker B. Francis Investigator in Anesthesiology. Presented in abstract form at the American Society of Anesthesiologists Annual Meeting, 1989, New Orleans, Louisiana.

Address reprint requests to Dr. Hansen: Department of Anesthesia, University of Iowa Hospitals and Clinics, GH6SE, Iowa City, Iowa 52242.

GENERAL ANESTHETICS are believed to act nonspecifically on the central nervous system (CNS) primarily through interaction of the anesthetic with lipid membranes, and the correlation between anesthetic potency and lipid solubility suggests a common, nonselective mechanism of anesthesia.[1,**] However, volatile anesthetics have differing abilities to alter physiologic parameters such as blood pressure, respiration, and analgesia, suggesting that some aspects of anesthesia may be mediated through interaction of the anesthetic with specific sites within the central nervous system. One such interaction may involve alteration of neurotransmitter binding,[2–5] turnover rates,[6,7] or metabolism.[8]

Of the many neurotransmitters in the CNS, the relationship between γ-aminobutyric acid (GABA) and inhalational anesthetics has received particular attention. Inhalational anesthetics have been shown to effectively increase the concentration of GABA within the synapse through inhibition of GABA catabolism.[8] This finding has led one group to hypothesize that increased concentrations of this inhibitory neurotransmitter within the synapse may contribute to a state of anesthesia.[9]

Benzodiazepine receptors in the CNS are linked to GABA receptors through a chloride coupling unit, such that benzodiazepine agonists increase the affinity of the γ-aminobutyric acid A ($GABA_A$) receptor for GABA ligands.[10,11] Given the interaction between inhalational anesthetics with GABA described previously,[8] as well as the reduction in inhalational anesthetic requirements with coadministration of benzodiazepines,[12] we hypothesized that anesthetics may interact with the benzodiazepine receptor, independently of direct anesthetic effects on the GABA receptor. We subsequently sought to explore the interaction of inhalational anesthetics with the benzodiazepine receptor in the rat using both *in vivo*[13] and *in vitro*[14,15] autoradiographic receptor binding techniques.

** Meyer KH: Contributions to the theory of narcosis. Trans Faraday Soc 33:1062–1068, 1937.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

Materials and Methods

In Vitro Autoradiography

The study was approved by our institutional Animal Care and Use Committee. Four-week-old male Sprague-Dawley rats (Biolabs, St. Paul, MN) were chosen for study based on the previous finding that the animals in this age range are ontogenetically mature with respect to benzodiazepine receptor density and function.[15] The animals were permitted free access to food and water until the time of the study. They were then killed by cervical dislocation and decapitated, and the brains were rapidly removed and frozen in 2-methylbutane (−40° C). (Freezing has been shown to not alter the binding characteristics of the benzodiazepine receptor.[16]) Frozen brains were immediately cut into 10-μm-thick serial sagittal sections on a freezing microtome at −20° C. Four serial sections were taken at 150-μm intervals, mounted on glass slides, and dried under vacuum for 12 h at 0° C. Slide-mounted sections, stored overnight at 0° C with desiccant, were allowed to equilibrate to room temperature for a period of 40 min before tissue–ligand incubation.

Slides were preincubated in Tris-HCl buffer solution (Boehringer Biochemicals, Indianapolis, IN) (50 mM, *p*H 7.4) for 10 min at 37° C. To determine total binding, slide-mounted brain sections were transferred to a second incubation chamber containing 50 ml Tris buffer solution at 37° C. Each buffer solution contained one of six predetermined concentrations (0.5, 1.0, 2.0, 4.0, 8.0, and 12.0 nM) of [^{3}H]-Ro-15-1788 (specific activity: 74.9 mCi/mmol; New England Nuclear, Boston, MA). The [^{3}H]-Ro-15-1788 concentration in solution was verified by liquid scintillation counting.

Slide-mounted brain sections were then assigned to control, halothane, or isoflurane experimental groups. In control groups, air was gently introduced into the buffer solution through an airstone at a rate of 25 ml/min. In the halothane and isoflurane experimental groups, volatile anesthetic was delivered in air at a rate of 25 ml/min such that the final concentration of halothane in solution was 0.34 mM, whereas that of isoflurane was 0.33 mM. These values approximated those equivalent to 1 MAC levels for halothane and isoflurane in the rat[17] (1.05% or 1.38%, respectively) (fig. 1). Volatile anesthetic concentration in buffer solution at 37° C was verified with gas chromatography.

Anesthetic concentration in solution was determined before experimental trials in Tris (50 mM) at 10, 20, and 30 min after introduction of volatile anesthetic into solution. Equilibrium was achieved at the first time point measured (10 min). Total incubation time of brain sections in [^{3}H]-Ro-15-1788-labeled buffer solution was 50 min. Trials for a second group of control brain sections, in which only air (*i.e.*, no anesthetic) was introduced into the buffer solution, were performed as above, but with preincubation and incubation temperature adjusted to 4° C rather than 37° C. These trials were conducted to validate our technique by allowing comparisons with previous studies that have examined [^{3}H]-Ro-15-1788 binding at 4° C.[18] For all experimental conditions, trials were performed in triplicate.

After incubation in [^{3}H]-Ro-15-1788-labeled Tris-HCl buffer, slides were immersed for 5 min in room-temperature Tris-HCl buffer (*p*H 7.4, 50 mM) to reduce nonspecific binding. The slides were then briefly dipped in distilled water and dried under a stream of cool, dry air for 2 min. Slides were then affixed to a mounting board and exposed to Hyperfilm-[^{3}H]® (Amersham, Arlington Heights, IL) for 2 weeks.

To determine nonspecific binding, homologous assays were conducted under halothane, isoflurane, and control conditions at 37° C in the presence of 1.0 μM unlabeled clonazepam at each [^{3}H]-Ro-15-1788 concentration level described above. An additional control group was studied with temperature adjusted to 4° C in the presence of 1.0 μM unlabeled clonazepam.

In Vivo Autoradiography

Four-week-old male Sprague-Dawley rats (Biolabs) were permitted free access to food and water until the time of

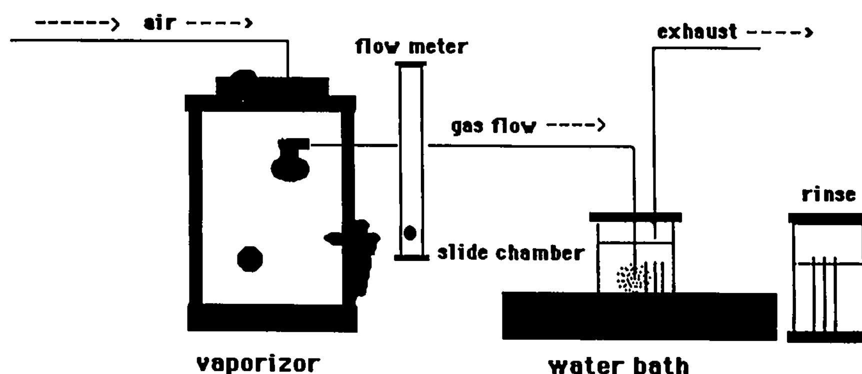

FIG. 1. System used for *in vitro* binding determinations. During halothane or isoflurane experimental trials, volatile anesthetics were vaporized and introduced *via* air into the slide incubation chamber. In control trials, the vaporizor was bypassed. Temperature within the incubation chamber was controlled with a water bath. After a 50-min incubation period, slide-mounted brain sections were washed 5 min in Tris buffer and rapidly rinsed in distilled water.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

the study. Animals were randomly assigned to halothane, isoflurane, or unanesthetized control groups. Animals in the anesthetic groups were anesthetized with 1.5% halothane or 2.0% isoflurane in 30% O_2/70% N_2. A tracheostomy tube was inserted and connected to a small animal ventilator set to deliver a tidal volume of 0.8 ml at a respiratory rate of 70 breaths per min. Femoral arterial and venous catheters were inserted through a surgical incision in the groin. The arterial catheter was connected to a pressure transducer for continuous blood pressure monitoring, whereas the venous catheter was used for drug and fluid administration. Heparin (20 IU) was administered intravenously to each rat.

Total surgical time was 35 min (measured from the start of induction). After surgery, inspired anesthetic agent concentration (measured with a Puritan Bennet/Datex® Model 222 Anesthetic Agent Analyzer) was reduced to either 1 MAC isoflurane (1.38%) or 1 MAC halothane (1.05%) in 33% O_2/64% N_2.[17] Rats remained anesthetized an additional 55 min. Arterial blood gases were measured 45 min after induction and immediately before isotope infusion on an automated blood gas analyzer (Radiometer ABL-2®; Copenhagen, Denmark). The ventilator was adjusted to maintain normoxia (Pa_{O_2} = 110–140 mmHg) and normocarbia (Pa_{CO_2} = 36–44 mmHg). Blood withdrawn for arterial blood gas sampling was replaced with rat donor blood on a volume-by-volume basis. Mean arterial pressure (MAP) was maintained within the range of 65–80 mmHg by infusion of rat donor blood. Rectal temperature was monitored with a pediatric temperature probe and maintained within the range of 36.5–37.5° C by surface heating or cooling.

Control animals were anesthetized with 2.0% halothane in 30% O_2/70% N_2 by a snout mask. A femoral venous catheter was inserted through a surgical incision. The venous catheter was used for drug and fluid administration. Heparin (20 IU) was administered intravenously to each rat. The venous catheter was capped and the wound site sutured closed. Anesthesia was discontinued and animals were returned to their cages, breathing spontaneously. A light plaster cast was applied to the animal's lower torso to prevent injury to the catheter site.

After a 55-min exposure period to 1 MAC of either halothane or isoflurane in the anesthetic groups or a 24-h postsurgical recovery interval in the control group, 0.5 μCi/g [^{3}H]-Ro-15-1788 (specific activity 74.9 mCi/mmol, New England Nuclear) was infused intravenously at a constant rate over 30 s to each rat. The animals were killed by decapitation 15 min later. MAP was continuously monitored during this time in the halothane and isoflurane groups, but not in controls. Nonspecific binding was assessed by pretreating a separate group of animals with clonazepam (5 mg/kg intraperitoneally) 30 min before [^{3}H]-Ro-15-1788 infusion.

After decapitation, brains were rapidly removed (less than 2.5 min) and frozen in 2-methylbutane (−40° C). Brains from five halothane-treated, five isoflurane-treated, and five awake control animals were analyzed. Frozen brains were cut in 10-μm serial sagittal sections on a cryostat at −20° C. Quadruplicate sections taken at 150-μm intervals were mounted on glass slides and exposed to Hyperfilm-[^{3}H]® in an x-ray cassette for 6 weeks, along with 16 [^{3}H] standards (American Radiolabeled Chemicals, St. Louis, MO).

AUTORADIOGRAPHY AND IMAGE ANALYSIS

Quadruplicate images of seven anatomically standardized brain sections from each animal in the *in vivo* experiment, as well as quadruplicate images of seven standardized brain sections from the halothane-treated, isoflurane-treated, and control groups at each concentration level (0.5–12 nM) in the *in vitro* study, were chosen for additional analysis based on standardized anatomic landmarks. These autoradiographic images were converted to digitized optical density images on a scanning microdensitometer system (Eikonix, Kodak, Bedford, MA) with a camera aperture of 100 μm and a fixed focal length. Optical densities from these autoradiographic images and standard radioactivity values in tissue equivalents derived from ^{3}H standards were entered into a Digital Micro-Vax II computer system. Calculation of bound ligand was determined by converting radioactivity values in brain images to bound [^{3}H]-Ro-15-1788 (femtomoles per milligram tissue) values with the use of the specific activity of the ligand (74.9 mCi/mmol).

IN VITRO AUTORADIOGRAPHY DATA ANALYSIS

Individual digitized [^{3}H]-Ro-15-1788 images at each concentration level (0.5–12 nM) were pseudo-color enhanced and displayed on a cathode ray screen. Total [^{3}H]-Ro-15-1788 bound in cortical brain regions was determined at each concentration level within each experimental group. Nonspecific binding values were similarly determined at each respective concentration level from images of brain sections pretreated with clonazepam. Specific binding was determined by subtracting mean nonspecific binding values from total binding values.

To determine maximum bound ligand per milligram of tissue (B_{max}) and the dissociation constant (K_D) for each experimental group (halothane, isoflurane, and unanesthetized), a Scatchard plot of *in vitro* data was constructed. Mean bound ligand (B) values at each concentration level within each experimental group were plotted on the y-axis *versus* corresponding mean bound/free (B/F) ligand values on the x-axis. Best fit lines were determined for all experimental groups from B and B/F data points with the use of a linear least-squares fit. Slopes (K_D) and y-

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

intercepts (B_{max}) for each inhalational group were compared through interaction between B and B/F values with the use of a regression analysis with indicator variables.[19]

In Vivo Autoradiography Data Analysis

Individual digitized [^{3}H]-Ro-15-1788 images were pseudo-color-enhanced and displayed on a cathode ray screen. With the use of an operator-controlled cursor, the cortical mantle was circumscribed and total bound ligand for that region recorded, along with the area of that region (in pixel units). To provide an estimate of global total cortical [^{3}H]-Ro-15-1788 binding, values from each section were area weighted and averaged over the seven sections in each rat. Nonspecific binding values were determined as above from brain images of rats pretreated with clonazepam. Specific binding was determined by subtracting mean nonspecific binding values from mean total binding values. Area weighted specific binding and mean physiologic values (Pa_{CO_2}, Pa_{O_2}, *p*H, MAP) were compared among anesthetic-treated and awake animals by one-way analysis of variance and a Newman-Keuls test for between-group differences where indicated by a significant F ratio.

[^{3}H]-Ro-15-1788 Pharmacokinetics

Fifteen male Sprague-Dawley rats (4 weeks of age) were randomly assigned to halothane-treated, isoflurane-treated, or control groups. Animals within each group were prepared in a manner identical to that described above (*In Vivo* Autoradiography) before injection of isotope. However, in contrast to the *in vivo* autoradiographic study, a femoral arterial catheter was placed in addition to the femoral venous catheter in awake control animals, and MAP was monitored continuously in all rats. An arterial blood gas sample was drawn immediately before isotope infusion; 0.25 μCi/g [^{3}H]-Ro-15-1788 (specific activity 74.9 mCi/mmol, New England Nuclear) then was infused intravenously at a constant rate over 30 s in each rat. Arterial blood samples (20 μl) were collected on chromatography paper at 3, 5, 9, 13, 15, and 20 min after injection of isotope. An additional blood sample (100 μl) was collected at the 15-min time point and centrifuged to permit determination of plasma radioactivity in each rat. Temperature was controlled (36.5–37.5° C) by surface heating or cooling. Twenty minutes after injection of the isotope, the animals were killed with halothane.

Timed arterial blood and plasma samples were air-dried, placed in scintillation vials, suspended in 1 ml distilled water, and digested with 240 μl NCS (Amersham) tissue solubilizer at 50° C for 20 min. Samples then were bleached with benzoyl peroxide at 50° C for 30 min, neutralized with 8 μl glacial acetic acid, and allowed to stand overnight at 50° C with 18 ml OCS scintillation cocktail. Blood and plasma samples were counted for ^{3}H isotope activity after 24 h on a Beckman Model 3801 scintillation counter. Counting efficiency was 40% for arterial samples and 45% for plasma samples. Individual arterial concentration–time curves were fitted through nonlinear regression analysis for each animal. The area under the arterial concentration–time curves (AUCs) was determined through integration of the resulting polynomial from time 0 to 20 min. Total drug clearance over this time period was estimated with the equation: dose/AUC = clearance.[20]

Mean drug clearance values from arterial data, mean plasma concentrations, and physiologic values were compared between anesthetic-treated and awake animals by a one-way analysis of variance. Specific intergroup differences were identified with the use of the Newman-Keuls test where indicated by a significant F ratio. Values throughout are reported as mean ± SD.

Results

In Vitro Autoradiography

Specific [^{3}H]-Ro-15-1788 binding was saturable (fig. 2), and maximal binding was obtained at 12 nM [^{3}H]-Ro-15-1788. Nonspecific binding, defined as binding in the presence of excess unlabeled ligand (clonazepam), was very low (specific:nonspecific ratio ~8:1 at 37° C, ~14:1 at 4° C). There were no differences in nonspecific binding among anesthetic-treated and control brains. Analysis of Scatchard data at 37° C demonstrated three lines; there was no significant difference in either K_D or B_{max} between anesthetic agents at 37° C (K_D: P = 0.32; B_{max}: P = 0.63), or between halothane- (K_D: P = 0.52; B_{max}: P = 0.21) or isoflurane- (K_D: P = 0.10; B_{max}: P = 0.64) treated groups compared with the non–anesthetic-treated group at 37° C (table 1). Scatchard analysis of control *in vitro* binding data at 4° C revealed a single line. Comparison between

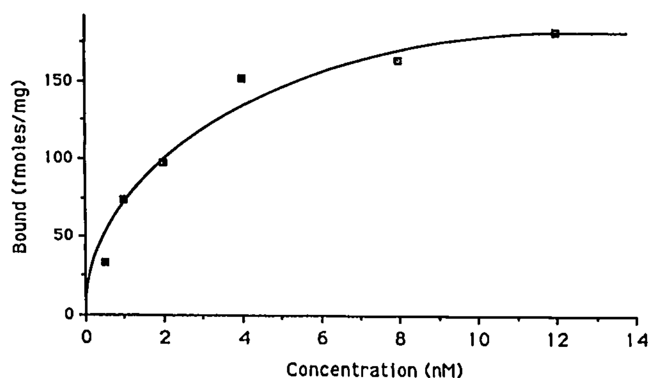

FIG. 2. *In vitro* saturation of [^{3}H]-Ro-15-1788–specific binding (femtomoles per milligram) in cortical brain tissue regions. Triplicate tissue samples were incubated in varying concentrations of [^{3}H]-Ro-15-1788 (0.5–12.0 nM) for 50 min at 37° C and processed as described in the text.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

TABLE 1. Experimental Data From *In Vitro* Scatchard Analysis

	Temperature (°C)	K_d (mM)	B_{max} (fmol/mg tissue)
Halothane	37.0	2.30	234.0
Isoflurane	37.0	2.42	226.0
Control (air)	37.0	2.17	221.0
Control (air)	4.0	0.81*	217.0

K_d and B_{max} values measured from the cortex of rat brain slices incubated in Tris buffer (50 m*M*, *p*H 7.4). Scatchard data represents mean cortical binding determined from triplicate tissue samples incubated in varying concentrations [^{3}H]-Ro-15-1788 (0.5–12.0 nM) and processed as described in the text. Intergroup comparisons were made using indicator variables.[19] *Significantly different ($P < 0.05$) from control values at 37° C.

non–anesthetic-treated groups at 4° C and 37° C *in vitro* revealed a significant difference in K_D ($P = 0.001$) but no difference in B_{max} ($P = 0.71$) (fig. 3).

In Vivo Autoradiography

Physiologic values determined before isotope infusion are reported in table 2. No significant differences were observed with respect to MAP, Pa_{CO_2}, Pa_{O_2}, *p*H, or body temperature between halothane- and isoflurane-treated groups. Because an arterial catheter was not placed in control animals, the above physiologic parameters are not available for this group.

Specific [^{3}H]-Ro-15-1788 cortical binding was not different between halothane- and isoflurane-anesthetized animals (table 2). However, cortical binding was greater in both anesthetic-treated groups compared with the unanesthetized group (halothane = 125 ± 23 fmol/mg; isoflurane = 130 ± 25 fmol/mg; unanesthetized = 80 ± 9 fmol/mg). There was no difference between groups with respect to nonspecific binding.

Kinetic Data

Physiologic values determined before isotope infusion are reported in table 3. No significant intergroup differences were observed with respect to MAP, Pa_{CO_2}, Pa_{O_2}, *p*H, body temperature, or animal weight.

Analysis of [^{3}H]-Ro-15-1788 clearance data over 20 min demonstrated no difference between halothane- and isoflurane-anesthetized animals (table 3). However, control animals showed a higher rate of drug clearance over 20 min compared with halothane- ($P < 0.05$) and isoflurane- ($P < 0.05$) anesthetized animals (halothane = 6.46 ± 0.40 $ml \cdot kg^{-1} \cdot min^{-1}$; isoflurane = 6.42 ± 0.61 $ml \cdot kg^{-1} \cdot min^{-1}$; unanesthetized = 7.64 ± 0.53 $ml \cdot kg^{-1} \cdot min^{-1}$). The [^{3}H]-Ro-15-1788 plasma values at 15 min were accordingly higher in halothane- ($P < 0.05$) and isoflurane- ($P < 0.05$) anesthetized animals compared with controls (halothane = $8.13 \pm 0.86 \cdot 10^{-7}$ mg/ml; isoflurane = $7.91 \pm 1.0 \cdot 10^{-7}$ mg/ml; and control = $6.14 \pm 0.53 \cdot 10^{-7}$ mg/ml).

Discussion

Administration of benzodiazepines in conjunction with volatile anesthetics has been shown to reduce volatile anesthetic requirements in humans[21] and animals.[12] Diazepam and midazolam infusions have reduced halothane MAC requirements in a dose-related manner, decreasing MAC levels by 73 and 30%, respectively.[22,23] Furthermore, administration of flumazenil (Ro-15-1788), a benzodiazepine antagonist, partially reversed the effects of isoflurane on EEG and measurements of cerebral metabolic rate for O_2 (CMR_{O_2}).[24]

This information, coupled with accounts of increased GABA concentrations in the presence of volatile anesthetics,[8] raised speculation that volatile anesthetics may in part interact through the benzodiazepine–GABA receptor complex. In this study, we have examined the effects of volatile anesthetics on [^{3}H]-Ro-15-1788 *in vitro* and *in vivo* receptor binding. *In vitro* halothane and isoflurane did not alter receptor affinity (K_D) or the number of occupied receptors (B_{max}) when compared with control brain sections. *In vivo*, the volatile anesthetics apparently enhanced binding compared with in unanesthetized animals. Recognizing this discrepancy, we performed the kinetic study and found reduced [^{3}H]-Ro-15-1788 ligand clearance, resulting in increased blood concentrations of ligand in anesthetic-treated animals. Therefore, the enhanced *in vivo* binding observed during anesthesia can be explained at least in part by differences in ligand availability to cerebral tissue. The results of this study thus indicate no direct interaction between volatile anesthetics and the benzodiazepine receptor site.

Several methodologic aspects of our autoradiographic

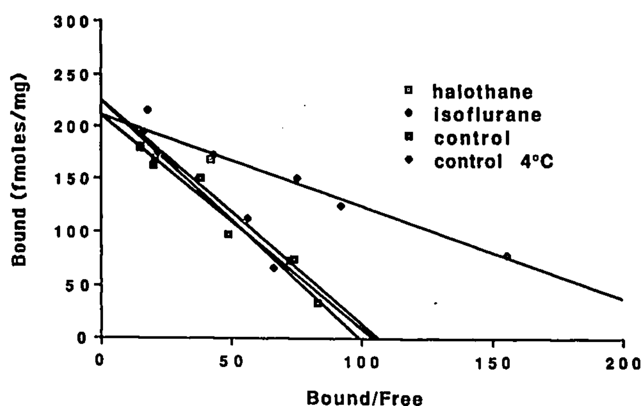

FIG. 3. Scatchard analysis of specific binding (femtomoles per milligram) measured in the cortex of rat brain autoradiographic images. [^{3}H]-Ro-15-1788 (0.5–12 nM) was incubated in triplicate with or without excess unlabeled clonazepam. Specific binding was the total binding minus that not displaced by unlabeled clonazepam. Linear regression analysis of the data in the Scatchard plot indicated the following values: halothane 37° C: K_d = 2.30, B_{max} = 234, r = 0.96; isoflurane 37° C: K_d = 2.42, B_{max} = 226, r = 0.97; control 37° C: K_d = 2.17, B_{max} = 221, r = 0.98; control 4° C: K_d = 0.81, B_{max} = 217, r = 0.92.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

TABLE 2. *In Vivo* Physiologic and Autoradiographic Data

	Halothane (n = 5)	Isoflurane (n = 5)	Awake (n = 5)
Physiologic data			
Body weight (g)	45 ± 2	48 ± 2	45 ± 1
MAP (mmHg)	76 ± 6	81 ± 7	NM
Pa_{CO_2} (mmHg)	38 ± 3	38 ± 4	NM
Pa_{O_2} (mmHg)	153 ± 37	125 ± 26	NM
Autoradiographic data			
[^{3}H]-Ro-15-1788 specific cortical binding (fmol/mg)	125 ± 23*	130 ± 25*	80 ± 9
Nonspecific cortical binding (fmol/mg)	13 ± 6	15 ± 8	13 ± 6

Physiologic values (mean ± SD) were determined from arterial blood gas samples measured immediately prior to isotope infusion. NM = value not measured in this group.

Experimental data from *in vivo* autoradiographic analysis. [^{3}H]-Ro-15-1788–specific cortical binding (femtomoles per milligram) and nonspecific cortical binding determined from seven quadruplicate brain sections in animals decapitated 15 min after isotope infusion. *$P < 0.05$ compared to control (nonanesthetized) group.

studies as well as our kinetic analysis must be addressed. The *in vivo* autoradiographic study involved examination of binding characteristics at one time point (15 min) after administration of an intravenous dose of [^{3}H]-Ro-15-1788. *In vivo,* the density and pattern of [^{3}H]-Ro-15-1788 binding in the CNS is changed by tissue perfusion over time, eventually favoring those tissue sites that have demonstrated the highest density of benzodiazepine receptor sites *in vitro.*[25] Thus, the analysis of binding at one time point may not completely reflect the entire history of the ligand. However, previous *in vivo* techniques using [^{3}H]-Ro-15-1788 examined binding over time and chose that point that most closely reflected the distribution in *in vitro* autoradiographic studies at 1.0 nM for subsequent trials.[13] In our analysis of *in vivo* Ro-15-1788 binding, a 15-min time point after injection closely reflected the distribution of [^{3}H]-Ro-15-1788 binding *in vitro.* This was determined by comparing the ratio, or relative distribution, of binding within the brain *in vivo* with the binding *in vitro* at the 1.0 nM concentration level. These values correlated well (cortex/caudate: *in vivo* = 6.5, *in vitro* = 6.3; cortex/hippocampus: *in vivo* = 1.2, *in vitro* = 1.1).

TABLE 3. [^{3}H]-Ro-15-1788 Kinetic Analysis

	Halothane (n = 5)	Isoflurane (n = 5)	Awake (n = 5)
Physiologic data			
Animal weight (g)	50 ± 5	51 ± 2	46 ± 4
MAP (mmHg)	70 ± 2	72 ± 2	78 ± 9
Pa_{CO_2} (mmHg)	41 ± 4	40 ± 4	37 ± 2
Pa_{O_2} (mmHg)	124 ± 20	116 ± 13	96 ± 8
*p*H	7.39 ± .03	7.39 ± .02	7.37 ± .0
Transfused blood volume (ml)	.50 ± .20	.45 ± .15	.53 ± .20
Kinetic data			
Clearance (20 min) (ml/kg/min)	6.46 ± .40*	6.42 ± .61*	7.64 ± .53
Plasma [^{3}H]-Ro-15-1788 ($\times 10^{-7}$ mg/ml)	8.13 ± .86*	7.91 ± 1.0*	6.14 ± .55

Physiologic values (mean ± SD) determined from arterial blood gas samples measured immediately prior to infusion of [^{3}H]-Ro-15-1788. There were no differences among groups. Transfused blood volume represents mean volume of donor rat blood infused prior to injection of isotope.

Experimental data from kinetic analysis. Clearance values (milliliters per kilogram per minute) for Ro-15-1788 determined over 20 min from timed arterial blood samples. [^{3}H]-Ro-15-1788 plasma values ($\times 10^{-7}$) were determined at the 20-min time point after injection of isotope.

* $P < 0.05$ compared to control (nonanesthetized) group.

In both the *in vivo* autoradiographic study and the kinetic analysis, casted spontaneously breathing animals were compared with anesthetized, mechanically ventilated animals; both mechanical ventilation[26] and externally applied pressure (casting) may alter central venous return, thereby altering drug distribution and subsequently altering [^{3}H]-Ro-15-1788 binding in the CNS as a result of factors independent of a direct anesthetic effect. Furthermore, inhalational anesthetics themselves modify drug clearance through alteration of hepatic blood flow,[27] adding yet another confounding variable to the direct assessment of *in vivo* binding in the presence of volatile anesthetics. To assess the cumulative effect of these factors on drug clearance from the blood, we evaluated the timed history of [^{3}H]-Ro-15-1788 in a separate group of rats by measuring blood radioactivity levels for a period of 20 min after intravenous injection. We observed a reduction in timed clearance of blood radioactivity, resulting in effectively increased blood levels, in anesthetized animals compared with unanesthetized animals. We acknowledge that this measurement was not performed as a formal determination of [^{3}H]-Ro-15-1788 pharmacokinetics, but rather as a way of approximating the effect volatile anesthetics may have on Ro-15-1788 plasma concentrations over the time course of the *in vivo* binding study. Because of the comparatively long plasma half-life of Ro-15-1788 (60 min),[28] errors assumed in measuring total blood ^{3}H activity as a reflection of the parent compound should be minimal.

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

In the *in vivo* autoradiographic study, no values for MAP, Pa_{O_2}, Pa_{CO_2}, or *p*H were available for awake animals. An arterial catheter, on the other hand, with measurement of the above values, was inserted in anesthetized animals. We justified this disparity on the basis that previous *in vivo* autoradiographic studies had not used invasive pressure monitoring or close measurement and control of arterial blood gases during the course of the experiment.[13,25] An arterial catheter was inserted in anesthetized animals to ensure normal MAP, normoxia, and normocarbia during the anesthetic; only after analysis of *in vivo* binding data was it apparent that alteration of physiologic parameters, such as MAP, hepatic blood flow, or drug clearance, may have contributed to apparent differences in receptor binding levels. Therefore, a "kinetic study" was performed in which animals were prepared identically to those in the *in vivo* autoradiographic study in an effort to identify measurable physiologic differences between awake and anesthetized animals that may have contributed to observed differences in binding. Because there was no statistical difference among the groups with respect to MAP, Pa_{O_2}, Pa_{CO_2}, or *p*H in the kinetic study, in which animals were prepared identically to those in the *in vivo* autoradiographic study, differences in these parameters among experimental groups in the original *in vivo* autoradiographic study seem unlikely.

The *in vitro* autoradiographic study involved incubating brain slices in buffer at 37° C while air was introduced into solution with or without volatile anesthetic. This design is somewhat atypical of *in vitro* binding studies, on two points. First, benzodiazepine binding is typically determined at 4° C, rather than 37° C, to improve specific/nonspecific binding ratios.[10] Despite this, we chose an *in vitro* incubation temperature of 37° C to more closely correlate with *in vivo* experimental conditions. However, to validate our technique with other studies that have examined [^{3}H]-Ro-15-1788 binding at 4° C, we performed a parallel analysis of non–anesthetic-treated brain sections at 4° C. A Scatchard plot of these results revealed a K_D and B_{max} of 0.93 nM and 210 fmol/mg tissue, respectively, which is in close agreement with previously published results at this temperature.[18] Furthermore, in non–anesthetic-treated brain sections, we have demonstrated a temperature-dependent reduction in binding affinity without alteration of B_{max}, which is typical of the benzodiazepine receptor.[29] Secondly, gas is not typically introduced to the incubation medium during binding studies. However, this technique has been used previously in opiate binding studies as a method to bring anesthetic gases into buffer solution,[3] without apparent alteration of K_D or B_{max}. Furthermore, as mentioned above, K_D and B_{max} determined in our study at 4° C closely reflect those values reported in binding studies that have not introduced gas into the incubation medium.[18]

In addition to the effects of temperature and volatile gases on *in vitro* receptor binding characteristics, endogenous compounds within the brain slices themselves may alter benzodiazepine binding. Because of the close correlation of benzodiazepine binding distribution in the brain to GABA-ergic synapses[30,31] and the enhancement of benzodiazepine binding in the presence of GABA *in vitro*,[32,33] the presence of endogenous GABA in brain tissue could affect benzodiazepine binding. Furthermore, inhalational anesthetics effectively increase GABA levels within the synapse *in vitro* by reducing GABA catabolism.[8] Therefore, alteration of benzodiazepine binding in the presence of volatile anesthetics may be a reflection of GABA levels, rather than an interaction of the anesthetic at the benzodiazepine receptor. However, in contrast to other benzodiazepine ligands, Ro-15-1788 binding is not affected by GABA[32] and therefore is independent of confounding influences of GABA in binding studies. Recent information presented by Miller *et al.*, by contrast, suggests GABA-ergic enhancement of benzodiazepine binding *in vivo* may occur with Ro-15-1788[34]; this is not conclusive, however. The possibility of a discrepancy in *in vivo* and *in vitro* binding data resulting from GABA-ergic enhancement of benzodiazepine binding remains, yet cannot be completely dismissed without the direct measurement of GABA concentrations *in vivo*.

The results of this experiment are consistent with other reports in the literature. Recent studies by Greiner and Larach,[35] as well as Schweiger *et al.*,[36] have also suggested the lack of volatile anesthetic activity at the benzodiazepine receptor as inferred by measurement of MAC levels of volatile anesthetics in the presence or absence of flumazenil (Ro-15-1788). This, of course, does not rule out the possibility that the GABA–benzodiazepine receptor complex is influenced by volatile anesthetics at sites within the complex other than the benzodiazepine receptor. In fact, recent evidence has demonstrated anesthetic perturbation of the chloride ion channel within the GABA–benzodiazepine receptor complex.[37] The interaction of volatile anesthetics at the chloride ion channel, with no direct effect on the benzodiazepine receptor itself, suggests a more specific mode of anesthetic action than that expected through general solubilization of lipid membranes, as suggested by the unitary theory of narcosis. Such information lends support to the premise that hydrophobic proteins or boundary lipids may be the primary sites of volatile anesthetic effect.[33,38,39]

Therefore, in contrast to previous studies that have speculated a possible effect of volatile agents at the benzodiazepine receptor,[24,40] we have currently demonstrated no direct interaction in the cortical mantle of the rat at physiologic temperature (37° C) *in vitro*. Enhanced cortical binding *in vivo* in the presence of volatile anesthetics as demonstrated in this study can be attributed in part to increased blood levels of ligand secondary to reduced Ro-15-1788 clearance during anesthesia. GABA-ergic enhancement of benzodiazepine binding *in vivo*, which cannot be excluded completely, may have also contributed

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022

to observed differences in *in vivo* and *in vitro* binding. We conclude that volatile anesthetics exert no direct action at the benzodiazepine receptor *in vivo* or *in vitro*.

The authors thank Twyla Salisbury for expert secretarial assistance and John Tinker, M.D., for continuing support.

References

1. Tanifuji Y, Eger EI, Terrel RC: Some characteristics of an exceptionally potent inhaled anesthetic: Thiomethoxyflurane. Anesth Analg 56:387–390, 1972
2. Lawrence D, Livinston A: Opiate-like analgesic activity in general anesthesia. Br J Pharmacol 73:435–442, 1981
3. Daras C, Cantrill RC, Gillman MA: ^{3}H naloxone displacement: Evidence for nitrous oxide as an opioid receptor agonist. Eur J Pharmacol 89:177–178, 1983
4. Inoki R, Kim SY, Maeda S, Nakamae J, Mishimo T, Yoshiya I: Effect of inhalational anesthetics on the opioid receptors in the rat brain. Life Sci 33(Suppl I):223–226, 1983
5. Labella F: Opiate radioreceptor assay distinguishes between anesthetics and convulsants: The effect of pH. Brain Res 241: 378–381, 1982
6. Ngai SH, Cheney DL, Finck DA: Acetylcholine concentrations and turnover in rat brain structures during anesthesia with halothane, enflurane, and ketamine. ANESTHESIOLOGY 48:4–10, 1978
7. Trabuchi M, Chaney L, Racagni G, Costa E: Pentobarbital and *in vivo* turnover rate of acetylcholine in mouse brain and in regions of rat brain. Pharmacol Res Commun 7:81–94, 1975
8. Cheng SC, Brenner EA: Inhibition of GABA metabolism in rat brain slices by halothane. ANESTHESIOLOGY 55:26–33, 1981
9. Cheng SC, Brenner EA: A neurochemical hypothesis for halothane anesthesia. Anesth Analg 54:242–246, 1975
10. Richards JG, Mohler H: Benzodiazepine receptors. Neuropharmacology 23:233–242, 1984
11. Haefely W: The biological basis of benzodiazepine actions. The benzodiazepines today—two decades of research and clinical experience. J Psychoactive Drugs 15:19–39, 1983
12. Hall RI, Schweiger IM, Hug CC: The anesthetic efficacy of midazolam in the enflurane-anesthetized dog. ANESTHESIOLOGY 68:862–866, 1988
13. Goeders NE, Kuhar MJ: Benzodiazepine receptor binding *in vivo* with [^{3}H]-Ro-15-1788. Life Sci 37:345–355, 1985
14. Young SW, Kuhar MJ: Radiohistochemical localization of benzodiazepine receptors in rat brains. J Pharmacol Exp Ther 212: 337–345, 1980
15. Braestrup C, Nielsen M: Ontogenetic development of benzodiazepine receptors in the rat brain. Brain Res 147:170–173, 1978
16. Braestrup C, Squires RF: Specific benzodiazepine receptors in rat brain characterized by high affinity [^{3}H] diazepam binding. Proc Natl Acad Sci USA 74:3805–3809, 1977
17. White PF, Johnston RR, Eger EI: Determination of anesthetic requirement in rats. Anaesthesia 40:52–57, 1974
18. McCabe RT, Olsen RW, Yezuita JP, Wamsley JK: Osmotic shock: A method to eliminate endogenous gamma-aminobutyric acid and account for the influence on benzodiazepine binding affinity in autoradiographic studies. J Pharmacol Exp Ther 245:342–349, 1988
19. Neter J, Wasserman W, Kutner MH: Applied Linear Regression Models. Homewood, Richard D. Irwin, 1983, pp 328–339
20. Altmayer P, Buch U, Hutschenreuter K, Buch HP: The volatile anesthetics, halothane, enflurane and isoflurane, influence the distribution of thiopental in man differently. Acta Anaesthesiol Scand 31:756–761, 1987
21. Perisho JA, Buechel DR, Miller RD: The effect of diazepam (Valium) on minimal alveolar anaesthetic requirement (MAC) in man. Can Anaesth Soc J 18:536–540, 1971
22. Tsunoda Y, Hattoyi Y, Takatsuka E, Sawa T, Hori T, Ikezano E: The effects of hydroxyzine, diazepam, and pentazocine on halothane minimum alveolar concentration. Anesth Analg 52: 390–394, 1973
23. Melvin MA, Johnson HB, Quasha AL, Eger EI: Induction of anesthesia with midazolam decreases halothane MAC in humans. ANESTHESIOLOGY 57:238–241, 1982
24. Roald OK, Forsman M, Steen PA: Partial reversal of the cerebral effects of isoflurane in the dog by the benzodiazepine antagonist flumazenil. Acta Anaesthesiol Scand 32:209–212, 1988
25. Samson Y, Hantraye P, Baron JC, Soussoline F, Comar D, Maziere M: Kinetics and displacement of ^{11}C Ro-15-1788, a benzodiazepine antagonist, studied in human brain *in vitro* by positron emission tomography. Eur J Pharmacol 110:247–251, 1985
26. Bastron RD: Hepatic and renal physiology, Anesthesia. Volume 2. Edited by Miller RD. New York, Churchill Livingstone, 1981, p 767
27. Epstein RM, Deutsch S, Cooperman LH, Clement AJ, Price HL: Splanchnic circulation during halothane anesthesia and hypercapnia in normal man. ANESTHESIOLOGY 27:654–661, 1966
28. Klatz U, Kanto J: Pharmacokinetics and clinical use of flumazenil (Ro-15-1788). Clin Pharmacokinet 14:1–12, 1988
29. Speth RC, Wastek GJ, Yamamura HI: Benzodiazepine receptors: Temperature dependence of [^{3}H] flunitrazepam binding. Life Sci 24:351–358, 1979
30. Olsen RW: Drug interaction at the GABA receptor–chloride ionophore complex. Annu Rev Pharmacol Toxicol 22:245–277, 1982
31. Unnerstall JR, Kuhar MJ, Niehoff DL, Palacios JM: Benzodiazepine receptors are coupled to a subpopulation of GABA receptors: Evidence from a quantitative autoradiographic study. J Pharmacol Exp Ther 218:797–804, 1981
32. Mohler H, Richards JG: Agonist and antagonist benzodiazepine receptor interaction *in vitro*. Nature 294:763–765, 1981
33. Mohler H, Richards JG, Wu JY: Autoradiographic localization of benzodiazepine receptors in immunocytochemically identifiable GABAergic synapses. Proc Natl Acad Sci USA 78:1935–1938, 1981
34. Miller LG, Greenblatt DJ, Barnhill JG, Summer WR, Shader RI: "GABA shift" *in vivo*: Enhancement of benzodiazepine-binding *in vivo* by modulation of endogenous GABA. Eur J Pharmacol 148:123–130, 1988
35. Greiner A, Larach D: The effect of benzodiazepine receptor antagonism by flumazenil on the MAC of halothane in the rat. ANESTHESIOLOGY 70:644–648, 1989
36. Schweiger IM, Szlam F, Hug CC: Absence of agonistic or antagonistic effects of flumazenil (RO-15-1788) in dogs anesthetized with enflurane, isoflurane, or fentanyl–enflurane. ANESTHESIOLOGY 70:477–480, 1989
37. Moody EJ, Suzdak PD, Paul SM, Skolnick P: Modulation of the benzodiazepine/gamma-aminobutyric acid receptor chloride channel complex by inhalational anesthetics. J Neurochem 51: 1386–1393, 1988
38. Evers AS, Berkowitz BA, d'Avignon DA: Correlation between anesthetic effect of halothane and saturable binding in brain. Nature 338:157–169, 1987
39. Franks NP, Lieb WR: What is the molecular nature of general anesthetic target sites? Trends Pharmacol Sci 8:169–174, 1987
40. Schwartz AE, Maneksha FR, Kanchugel MS, Sidhu US, Poppers PJ: Flumazenil decreases the minimum alveolar concentration of isoflurane in dogs. ANESTHESIOLOGY 70:764–766, 1989

Downloaded from http://asa2.silverchair.com/anesthesiology/article-pdf/74/1/97/320598/0000542-199101000-00016.pdf by guest on 24 January 2022