Pergamon

Biochemical Pharmacology, Vol. 49, No. 6, pp. 809–817, 1995.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00519-2

VOLATILE ANESTHETICS AND GLUTAMATE ACTIVATION OF *N*-METHYL-D-ASPARTATE RECEPTORS

DAN C. MARTIN,*†‡ MARC PLAGENHOEF,† JAMES ABRAHAM,*
ROBERT L. DENNISON*† and ROBERT S. ARONSTAM†§

*Department of Anesthesiology, Medical College of Georgia, Augusta, GA 30912; †Veterans Administration Medical Center, Augusta, GA 30910; and §Guthrie Research Institute, Sayre, PA 18840, U.S.A.

(*Received* 23 *April* 1994; *accepted* 4 *October* 1994)

Abstract—Several studies have indicated important functional interactions between volatile anesthetics and the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors. In the present study, we examined the effects of diethyl ether, chloroform, methoxyflurane, halothane, enflurane, and isoflurane on (1) glutamate activation of the NMDA receptor complex, including glycine reversal of anesthetic action, as revealed by [^{3}H] (5*R*,10*S*)-(+)methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine, dizocilpine (MK-801) binding to the cation channel, and (2) [^{3}H]*cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid (CGS 19755) binding to the glutamate recognition site of the NMDA receptor. In agreement with previous studies, glutamate increased the binding of 1 nM [^{3}H]MK-801, measured after a 1-hr incubation at 37°, by up to several hundred fold. This stimulation was blocked by glutamate antagonists and potentiated by glycine with an EC_{50} of ≈ 0.03 μM. Glycine also had a direct stimulatory effect on [^{3}H]MK-801 binding at much higher concentrations (≥ 10 μM). All of the anesthetics examined depressed glutamate stimulation of [^{3}H]MK-801 binding in a concentration-dependent manner with the following order of potency: halothane $\geq$ enflurane > methoxyflurane > chloroform > diethyl ether. This inhibition of [^{3}H]MK-801 binding was observed at concentrations that are routinely attained in the cerebrospinal fluid during surgical anesthesia. Moreover, the inhibition was reversed rapidly following removal of the anesthetics from the assay medium. Inclusion of glycine in the incubation medium markedly attenuated anesthetic-induced inhibition of glutamate-sensitive [^{3}H]MK-801 binding with an EC_{50} of between 0.1 and 1 μM. Thus, this reversal by glycine correlated with its potentiating, as opposed to its direct stimulatory, effect on NMDA receptors. Anesthetic inhibition of [^{3}H]MK-801 binding could not be overcome by raising the glutamate concentration (i.e. the interaction did not appear to be competitive with respect to glutamate) unless glycine was present. Binding of [^{3}H]CGS 19755 to the glutamate recognition site was also inhibited by each of the anesthetics examined. However, with the exception of chloroform, all of the anesthetics were more potent inhibitors of glutamate-stimulated [^{3}H]MK-801 binding than they were of [^{3}H]CGS 19755 binding. [^{3}H]CGS 19755 binding saturation curves in the presence of halothane and enflurane indicated a decrease in the density of [^{3}H]-CGS 19755 binding sites with no change in binding affinity (i.e. the inhibition did not appear to be competitive). These findings support the idea that anesthetic drugs disrupt NMDA receptor transmission through multiple allosteric effects on the receptor-channel activation mechanism and the glutamate binding site.

Key words: anesthetics; excitatory amino acid receptors; glutamate; glycine; halothane; *N*-methyl-D-aspartate receptors

NMDA‖ receptors are a class of glutamate receptors that mediate excitatory transmission in the central nervous system. NMDA receptors play a role in a number of important neurophysiological events, including epilepsy, memory, and ischemic neuronal toxicity [1]. Activation of NMDA receptors opens a non-selective cation channel that is characterized by high Ca^{2+} permeability, relatively slow kinetics, a large single channel conductance, and a voltage-dependent blockade by Mg^{2+} [2]. Glycine is an allosteric co-agonist at NMDA receptors, markedly potentiating receptor activation by glutamate [3]. MK-801 binds to a site within the NMDA ionophore to block glutamate or NMDA-stimulated ionic currents, while CGS 19755 is a competitive antagonist acting at the glutamate recognition site [4, 5]. The access of MK-801 to its site within the ion channel is sterically restricted unless the NMDA receptor is activated by an agonist. Thus, [^{3}H]MK-801 binding can be used as an indicator of channel opening. In the present study, [^{3}H]MK-801 and [^{3}H]CGS 19755 were used to determine the effects of several volatile anesthetics on NMDA receptor agonist binding and ion channel opening.

Several lines of evidence suggest interactions between volatile anesthetics and NMDA receptors: volatile anesthetic potency is increased by the NMDA receptor antagonists MK-801 [6, 7] and CGS 19755 [8], as well as by polyamines, a second class of positive, allosteric receptor modulators [9].

‡ Corresponding author: Dr. Dan C. Martin, Department of Anesthesiology, Medical College of Georgia, Augusta, GA 30912. Tel. (706) 721–4544; FAX (706) 721–7753.

‖ Abbreviations: NMDA, *N*-methyl-D-aspartate; MK-801, (5*R*,10*S*)-(+)methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]-cyclohepten-5,10-imine, dizocilpine; and CGS 19755, *cis*-4-(phosphonomethyl)piperidine-2-carboxylic acid.

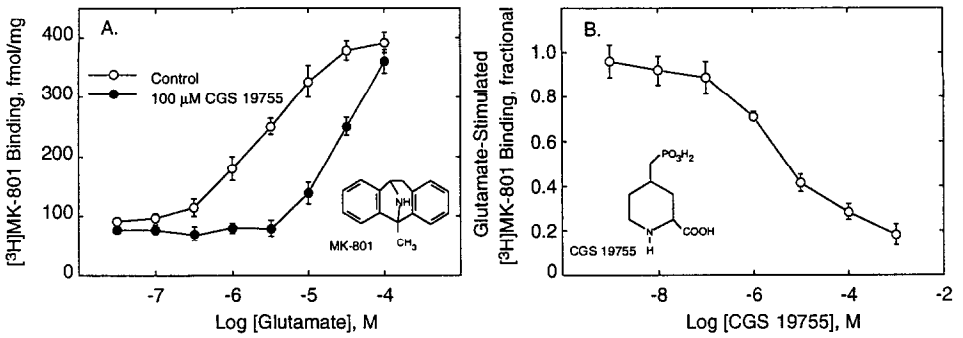

Fig. 1. Glutamate stimulation of [^{3}H]MK-801 binding. (A) The binding of 1 nM [^{3}H]MK-801 to membranes from rat cerebral cortex was measured in the presence of the indicated concentrations of glutamate. Binding was measured in the absence and presence of 100 μM CGS 19755, a competitive glutamate antagonist [4]. (B) Concentration dependence for CGS 19755 inhibition of the specific binding of 1 nM [^{3}H]MK-801 that is stimulated by 10 μM glutamate. Values are means ± SD from a series of 3 independent experiments performed in duplicate.

Isoflurane blocks NMDA-stimulated currents in cultured hippocampal neurons, decreasing both the frequency of channel opening and mean channel open time [10]. Moreover, it has been reported that volatile anesthetics specifically depress the glutamate-dependent intraneuronal translocation of calcium [11]. These latter two studies strongly suggest that a direct interaction between volatile anesthetics and NMDA glutamate receptors is responsible for the inhibition of channel activation. We have shown that enflurane disrupts glutamate-stimulated binding of [^{3}H]MK-801 to the NMDA ionophore, and that this disruption is attenuated by the positive NMDA receptor modulator glycine [12]. The present study was undertaken to ascertain: (1) the generality of these phenomena (i.e. anesthetic inhibition of NMDA channels and its reversal by glycine) with respect to several species of volatile anesthetics, (2) the reversibility of anesthetic action, (3) the relationship of anesthetic potency vis-à-vis NMDA receptors to their physicochemical properties, and (4) the effects of anesthetics on ligand binding to the glutamate recognition site.

MATERIALS AND METHODS

Tissue preparation. Membranes for radioligand binding assays were prepared as follows: adult, male Wistar rats (Harlan Sprague–Dawley, Indianapolis, IN) were killed by decapitation. Their cerebral cortices were homogenized at 2–4° in 5 mM Tris–HCl (pH 7.4). This homogenate was centrifuged at 17,000 *g* for 15 min. The pellet was resuspended in buffer and treated with Triton X-100 (0.04%) for 20 min at 37°, and then washed 4–5 times to remove the Triton and endogenous glutamate and glycine. Membranes used for [^{3}H]CGS 19755 binding assays were resuspended in 50 mM Tris–HCl buffer (pH 7.5). Those used for [^{3}H]MK-801 binding studies were resuspended in 5 mM Tris buffer. Protein concentration was determined by the method of Smith *et al.* [13].

Radioligand binding assays. [^{3}H]MK-801 (22.5 Ci/mmol; Dupont-NEN) binding was measured at 37° for 60 min in a final assay volume of 1 mL. Nonspecific binding was determined in the presence of 100 μM unlabeled MK-801. Membranes were collected on glass fiber filters by vacuum filtration using a Brandel Cell Harvester (Gaithersburg, MD), and radioactivity was measured by liquid scintillation counting using Scintiverse BD (Fisher Scientific).

[^{3}H]CGS 19755 (53.3 Ci/mmol; Dupont-NEN) binding assays were carried out at 37° for 20 min (preliminary experiments indicated that binding equilibrium was reached in this time) in a 1-mL assay volume containing 50 mM Tris–HCl, pH 7.4, and 200–300 μg membrane protein. Nonspecific binding was measured in the presence of 100 μM CGS 19755 (Ciba-Geigy). Membranes were collected by vacuum filtration and radioactivity was measured by liquid scintillation counting, as described above.

Anesthetics, with the exception of diethyl ether, were added to assay tubes as aliquots from stock anesthetic buffers. Diethyl ether was injected directly into the assay medium using a Hamilton syringe. Following agitation of the tubes, the membranes were added. During the course of a 60-min assay, the anesthetic concentration exposed to the membrane protein decreases by 10–15% [14]. The anesthetic concentrations reported were measured in the aqueous assay medium from sham assay tubes run in parallel with experimental tubes following 10 min of incubation at 37°. Anesthetic concentrations were determined by gas–liquid chromatography [14].

Data were compiled as mean [^{3}H]MK-801 or [^{3}H]-CGS 19755 binding values from multiple independent experiments, each performed in duplicate or triplicate. Binding parameters (B_{max} and K_D) were determined using nonlinear fits of saturation binding data to a model incorporating a single population of independent binding sites using the computer program Nonlin II (Stephen R. Ikeda, Medical College of Georgia).

RESULTS

Glutamate increased the binding of 1 nM [^{3}H]-

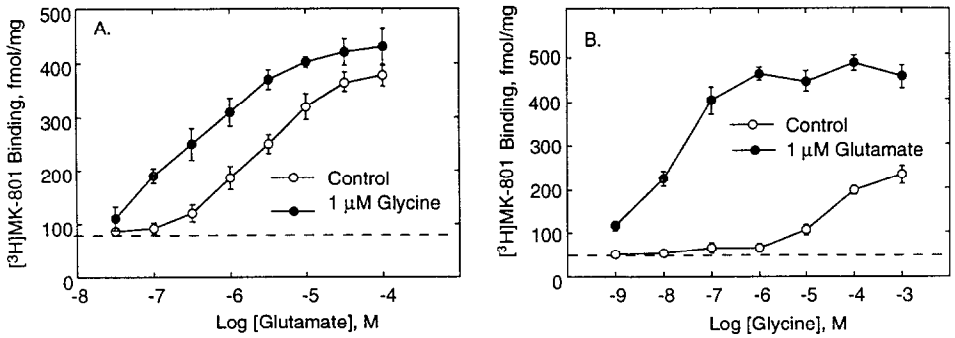

Fig. 2. Influence of glycine on glutamate stimulation of [^{3}H]MK-801 binding to NMDA receptors. (A) The binding of 1 nM [^{3}H]MK-801 to cortical NMDA receptors was measured in the presence of the indicated concentrations of glutamate in the absence and presence of 1 μM glycine. The dashed line indicates basal specific binding measured in the absence of glutamate or glycine. (B) The binding of 1 nM [^{3}H]MK-801 was measured in the presence of the indicated concentrations of glycine in the absence and presence of 1 μM glutamate. The dashed line indicates basal specific binding measured in the absence of glutamate or glycine. Values are means ± SD from 3 experiments.

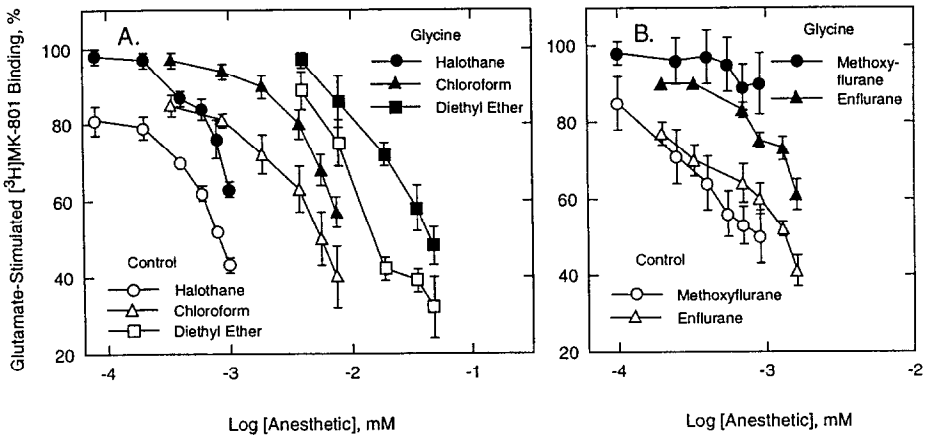

Fig. 3. Inhibition by volatile anesthetics of agonist-stimulated [^{3}H]MK-801 binding and reversal by glycine. Rat neural membranes were incubated with 1 nM [^{3}H]MK-801 in the presence of the indicated concentrations of halothane, enflurane, chloroform, methoxyflurane or diethyl ether in the presence of 100 μM glutamate (control; open symbols) or in the presence of 100 μM glutamate plus 100 μM glycine (glycine; closed symbols). Binding (mean ± SD; N = 4–6) is expressed as percent of specific binding measured in the absence of anesthetic, but in the presence of glutamate or glutamate plus glycine. Control binding was 920 fmol/mg protein in the presence of 100 μM glutamate and 1100 fmol/mg protein in the presence of 100 μM glutamate and 100 μM glycine.

MK-801 to cortical membranes after a 1-hr incubation at 37° by up to several hundred percent (Fig. 1A). The magnitude of this fractional stimulation in the different membrane preparations largely reflected the efficiency of the washing procedures in removing endogenous glutamate and/or glycine; the fractional stimulation was greatest in preparations in which basal [^{3}H]MK-801 binding was lowest, the level of total binding measured in the presence of saturating concentrations of glutamate and glycine being remarkably similar in all preparations. The EC_{50} for this stimulation by glutamate was in the range of 1–3 μM. CGS 19755, a potent competitive inhibitor of glutamate binding to the NMDA receptor [4], shifted the glutamate stimulation curve to the right (to EC_{50} ≈25 μM) in a manner consistent with competitive inhibition (Fig. 1A). Moreover, CGS 19755 decreased the stimulation of 1 nM [^{3}H]MK-801 binding by 10 μM glutamate to background levels with an IC_{50} of ≈3 μM (Fig. 1B).

Glycine, a positive NMDA receptor modulator, augmented glutamate stimulation of 1 nM [^{3}H]MK-801 binding (Fig. 2). This stimulation was most apparent in the presence of glutamate concentrations of less than 10 μM (Fig. 2A). The concentration–response relationship for glycine activation of NMDA receptors is shown in Fig. 2B. In this series of experiments, 1 μM glutamate increased specific [^{3}H]MK-801 binding by 120%. This stimulation was increased by glycine to a maximum of 487% at 1 μM

Table 1. Anesthetic inhibition of ligand binding to the NMDA receptor ion channel and glutamate recognition site

Anesthetic	IC_{40} (mM) [^{3}H]MK-801	IC_{30} (mM) [^{3}H]CGS 19775	Oil/gas partition coefficient (37°)
Halothane	0.48 ± 0.10	1.0 ± 0.2	224
Enflurane	0.66 ± 0.15	> 1.3	96.5
Isoflurane	ND*	> 0.7	90.8
Chloroform	4.4 ± 0.5	2.3 ± 0.5	265
Methoxyflurane	0.89 ± 0.07	0.8 ± 0.2	970
Diethyl ether	12 ± 2	50 ± 9	65

The concentrations of anesthetic that inhibited ligand binding by 40 or 30% are indicated. [^{3}H]MK-801 binding (1 nM) was measured in the presence of 100 μM glutamate with no exogenous glycine added. The concentration of [^{3}H]CGS 19775 was 20 nM. Oil/gas partition coefficients are as summarized by Koblin and Eger [15]. Values are means ± SD, N = 3.
*ND, not determined.

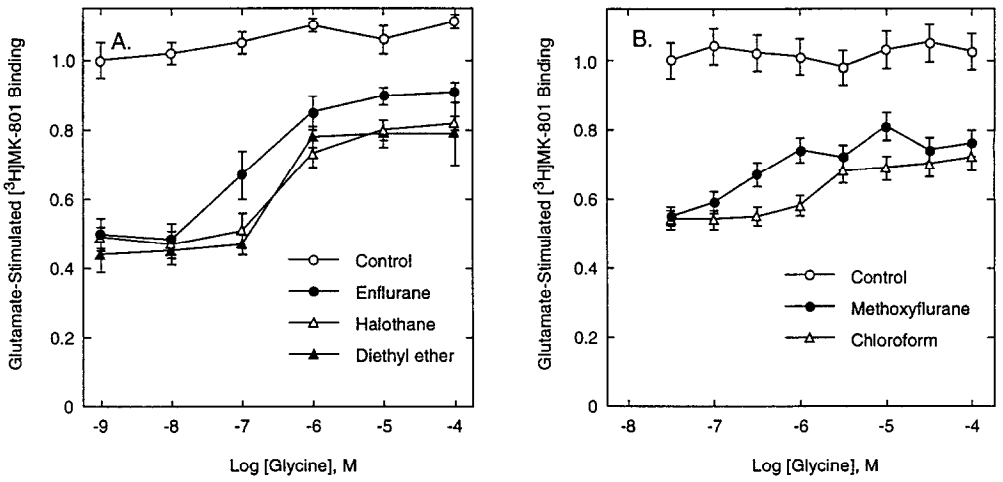

Fig. 4. Concentration dependence for glycine reversal of the inhibition of glutamate-stimulated [^{3}H]-MK-801 binding by volatile anesthetics. Rat cortical membranes were incubated with 1 nM [^{3}H]MK-801 in the presence of 100 μM glutamate in the presence or absence of the indicated anesthetics at a concentration sufficient to produce approximately 50% inhibition of [^{3}H]MK-801 binding (enflurane, 1 mM; halothane, 0.8 mM; diethyl ether, 35 mM; methoxyflurane, 1 mM; chloroform, 3.8 mM) and the concentrations of glycine indicated on the abscissa. Binding (mean ± SD; N = 4–6) is expressed as the fraction of specific binding measured in the absence of anesthetic.

glycine. The EC_{50} for this stimulation was ≈0.03 μM. In the absence of exogenous glutamate, glycine produced little stimulation of [^{3}H]MK-801 binding at concentrations below 10 μM (Fig. 2B). At high concentrations, however, glycine stimulated [^{3}H]-MK-801 binding by up to 150% (1 mM glycine in the absence of glutamate). The difference in the concentration dependencies for glycine stimulation of [^{3}H]MK-801 binding in the absence and presence of glutamate suggests that different mechanisms underlie the potentiating and direct stimulatory actions of glycine.

Halothane, enflurane, chloroform, methoxyflurane and diethyl ether inhibited glutamate-stimulated [^{3}H]MK-801 binding by at least 50% (Fig. 3). Comparing the anesthetics in terms of their IC_{40} concentrations, the following potency series was obtained: halothane ≥ enflurane > methoxyflurane > chloroform > diethyl ether. The IC_{40} values ranged from 0.48 to 12 mM (Table 1). Removal of the anesthetics from the assay medium by reduced pressure with agitation after a 10-min exposure to the membranes resulted in a return of [^{3}H]MK-801 binding to control levels.

The inhibition of glutamate-stimulated [^{3}H]MK-801 binding by each of the anesthetics was mitigated by glycine (Fig. 3). For example, the inhibition by enflurane in the range of 0.2 to 0.9 mM was eliminated completely by 100 μM glycine. Inhibition curves by chloroform, methoxyflurane and diethyl

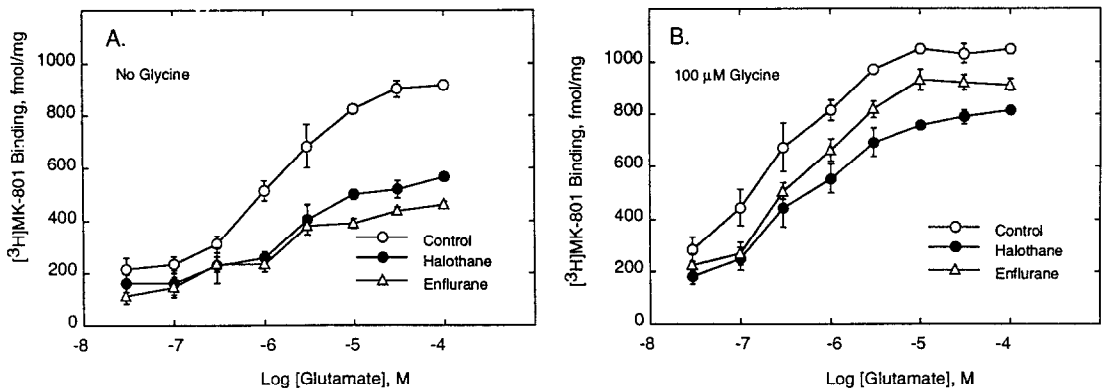

Fig. 5. Influence of halothane and enflurane on glutamate stimulation of [^{3}H]MK-801 binding. Cortical membranes were incubated in the absence (○) or presence of 0.8 mM enflurane (△) or 0.7 mM halothane (●) and the indicated concentrations of glutamate. The binding of 1 nM [^{3}H]MK-801 was measured at the end of a 1-hr incubation. Data represent mean binding data and SD from 4 experiments. Binding was measured in the absence (A) or presence (B) of 100 μM glycine.

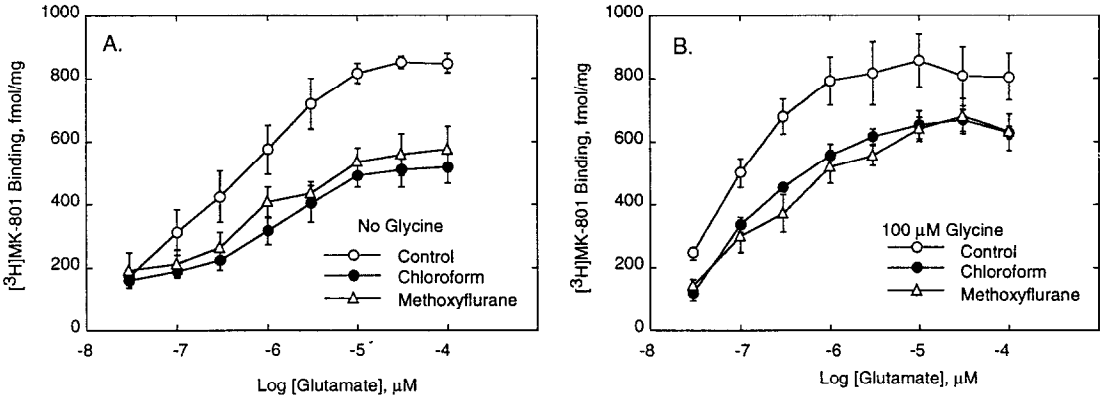

Fig. 6. Influence of chloroform and methoxyflurane on glutamate stimulation of [^{3}H]MK-801 binding. Cortical membranes were incubated in the absence (○) or presence of 1.1 mM methoxyflurane (△) or 3.8 mM chloroform (●) and the indicated concentrations of glutamate. The binding of 1 nM [^{3}H]MK-801 was measured at the end of a 1-hr incubation. Mean binding data and SD from 4 experiments are shown. Binding was measured in the absence (A) or presence (B) of 100 μM glycine.

ether were shifted to the right by a factor of 2- to 3-fold (Fig. 3B). The concentration dependence for glycine reversal of anesthetic inhibition of glutamate-stimulated [^{3}H]MK-801 binding is illustrated in Fig. 4. Using concentrations of anesthetic that inhibited stimulated binding by ≈50%, glycine was found to eliminate 60% of the inhibition produced by enflurane, halothane and diethyl ether with EC_{50} values between 0.1 and 1 μM (Fig. 4A). In these experiments, glycine was somewhat less effective at reversing the inhibition caused by methoxyflurane and chloroform (Fig. 4B). Note that the concentrations of glycine required to reverse anesthetic inhibition of stimulated [^{3}H]MK-801 binding correspond to the concentrations of glycine required to potentiate glutamate stimulation of [^{3}H]MK-801 binding, rather than the concentrations required to produce a direct stimulation of [^{3}H]MK-801 binding (compare Figs. 4 and 2B).

The influence of anesthetics on the concentration dependence of glutamate stimulation of [^{3}H]MK-801 binding in the absence and presence of glycine (100 μM) is illustrated in Figs. 5 and 6. At glutamate concentrations from 1 to 100 μM, the fractional inhibition of the stimulated binding by the anesthetic was fairly constant; however, in the absence of glycine, this fraction was ≈50%, whereas in the presence of glycine the fractional inhibition was 10–25%.

[^{3}H]CGS 19755 was used to label glutamate binding sites on the NMDA receptor. [^{3}H]CGS 19755 saturation curves performed in the absence and presence of halothane are shown in Fig. 7. In the absence of anesthetic, [^{3}H]CGS 19755 binding

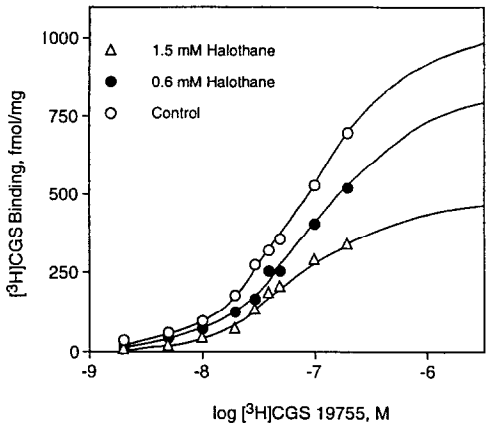

Fig. 7. Influence of anesthetics on [^{3}H]CGS 19755 binding saturation curves. Rat cortical membranes were incubated in the presence of the indicated concentrations of [^{3}H]CGS 19755 in the absence or presence of 0.6 mM or 1.5 mM halothane. Binding values are the means from triplicate determinations in a representative experiment. Lines are drawn according to parameters derived from nonlinear regression analyses using a model incorporating a single population of independent receptors.

was consistent with a single population of receptors with a density of 997 ± 29 fmol/mg protein and a dissociation constant of 87 ± 10 nM. Binding was decreased in the presence of halothane (Fig. 7). The apparent receptor density decreased to 789 ± 42 and 490 ± 42 fmol/mg protein in the presence of 0.6 and 1.5 mM halothane, respectively; there were no changes in the dissociation constants (96 ± 10 and 78 ± 28 nM, respectively). Thus, this inhibition is consistent with a noncompetitive interaction between halothane and [^{3}H]CGS 19755.

Inhibition of 20 nM [^{3}H]CGS 19755 binding by each of the anesthetics at 37° is shown in Fig. 8. Each of the anesthetics inhibited [^{3}H]CGS 19755 binding by at least 30%. Diethyl ether inhibited [^{3}H]CGS 19755 binding to the greatest extent, by up to 65% at high concentrations. The order of potency for inhibition of [^{3}H]CGS 19755 binding was similar to the potency for inhibition of [^{3}H]MK-801 binding, although the extent of the inhibition was less in each case (Table 1). Moreover, the potency of each anesthetic was less with respect to inhibition of [^{3}H]CGS 19755 binding compared with inhibition of [^{3}H]MK-801 binding.

DISCUSSION

Multiple effects of volatile anesthetics on NMDA receptors were revealed in radioligand binding assays using probes for the ion channel and agonist recognition sites: (1) glutamate stimulation of [^{3}H]-MK-801 binding was inhibited markedly by a series of volatile anesthetics in a noncompetitive manner; (2) glycine reversed most of the inhibition caused by the anesthetics; (3) inhibition by the anesthetics was readily reversed upon removal of the anesthetics from the incubation medium; (4) anesthetic inhibition occurred within concentration ranges achieved during surgical anesthesia; (5) anesthetics inhibited [^{3}H]CGS 19755 binding to the glutamate recognition site in a noncompetitive manner, although at somewhat higher concentrations and to a lesser extent than they affected ion channel activation.

Several lines of evidence suggest that blockade of NMDA receptors has an anesthetic effect. Volatile anesthetic potency is increased by the NMDA receptor antagonists MK-801 [6, 7] and CGS 19755 [8], as well as by polyamines [9]. Ketamine, a dissociative general anesthetic, blocks NMDA neurotransmission at clinically relevant concentrations [16, 17]. As with MK-801, ketamine blockade is voltage dependent and appears to occur at a site within the cation channel. Ethanol, in a manner analogous to the volatile anesthetics, disrupts

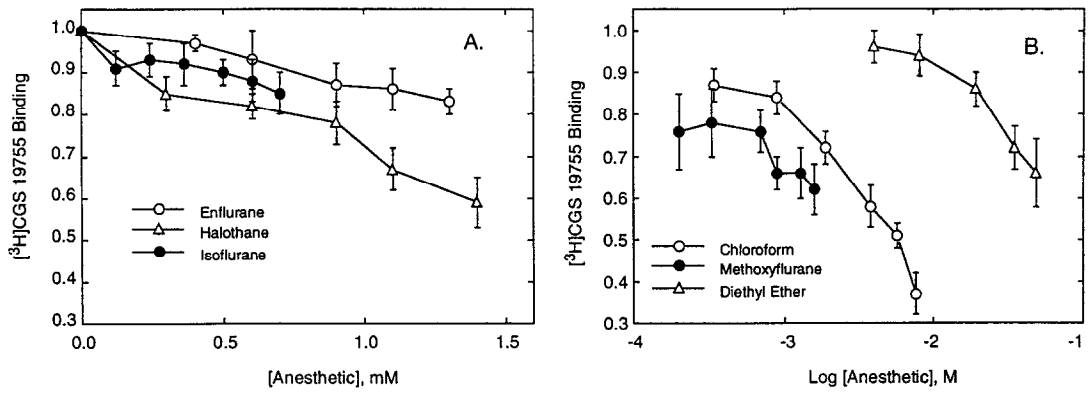

Fig. 8. Influence of volatile anesthetics on [^{3}H]CGS 19755 binding to NMDA receptors. The binding of 20 nM [^{3}H]CGS 19755 was determined after 20 min of incubation at 37° in cortical membranes exposed to the indicated concentrations of enflurane, halothane, isoflurane, chloroform, methoxyflurane or diethyl ether. Binding (mean ± SD, N = 5–7) is expressed as the fraction of total specific binding measured in the absence of anesthetic.

NMDA transmission by a process that is reversed by glycine [18]. Pentobarbital and secobarbital also disrupt ligand binding to the NMDA receptor as well as NMDA-stimulated $^{45}Ca^{2+}$ uptake by rat brain microvesicles [19]. It is interesting that other central nervous system depressants, such as pentobarbital, disrupt neurotransmission at other subtypes of glutamate receptor, including kainate and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors [20].

MK-801 binds to a site within the NMDA receptor ionophore, and access to this site in the absence of receptor agonists is sterically restricted. Thus, MK-801 blockade of NMDA-stimulated currents in electrophysiological experiments requires activation of the channel [21], and [^{3}H]MK-801 binding is greatly potentiated by receptor agonists (e.g. Refs. 22 and 23). Accordingly, the kinetics of [^{3}H]MK-801 binding are not simple. Bonhaus and McNamara [23] have argued that agonists increase the rates of [^{3}H]MK-801 binding association and dissociation without affecting the apparent affinity or steady-state level of binding; thus, basal [^{3}H]MK-801 binding was found to equal stimulated binding after a prolonged (8 hr) incubation at 25°. The present binding incubations were carried out at 37° for 1 hr. The elevated temperature was essential because of the strong dependence of anesthetic solubility (and, perhaps, activity) on temperature, solubility being much greater at lower temperatures. Incubations substantially longer than 1 hr at 37° led to a degradation of the receptor as indicated by a progressive decline in [^{3}H]MK-801 binding. Thus, the glutamate-stimulated increases in [^{3}H]MK-801 binding observed in the present experiments were accepted as empirical indications of receptor activation, and little theoretical significance was accorded to calculated binding parameters.

Site of anesthetic action. A fundamental question is where do volatile anesthetics act to disrupt transmission at NMDA receptors. This site could be within the cation channel, within the glutamate recognition site, within the glycine receptor, or at some other, undefined allosteric site.

The site of volatile anesthetic action does not appear to reside within the cation channel since (1) basal [^{3}H]MK-801 measured in the absence of glutamate was not inhibited significantly by any of the anesthetics (i.e. the inhibition was specific for agonist-stimulated binding), and (2) anesthetic inhibition observed in the presence of glutamate was diminished by the positive modulator glycine. To minimize the influence of endogenous agonists, the influence of anesthetics on basal [^{3}H]MK-801 binding was measured (in the absence of exogenous glutamate) in membrane preparations that were washed sufficiently to ensure a 400% increase in [^{3}H]MK-801 binding when stimulated by 100 μM glutamate. If the ionophore were the site of anesthetic action, similar degrees of inhibition would be expected no matter how the channel was opened, whether by glutamate alone or by glutamate plus glycine. The anesthetic action seems to be specific for the channel activation process that is stimulated by agonists and potentiated by glycine.

A second possibility is that the anesthetics directly interfere with glutamate binding to its recognition site on the receptor. However, most anesthetics were less potent inhibitors of [^{3}H]CGS 19755 binding than of glutamate-stimulated [^{3}H]MK-801 binding. Moreover, the extent of inhibition of [^{3}H]CGS 19755 binding was less than that of glutamate-stimulated [^{3}H]MK-801 binding. For example, enflurane was the most potent inhibitor of glutamate-stimulated [^{3}H]MK-801 binding but the least potent inhibitor of [^{3}H]CGS 19755 binding. In addition, [^{3}H]CGS 19755 saturation curves suggested a noncompetitive interaction between the anesthetics and [^{3}H]CGS 19755. Moreover, increasing concentrations of glutamate failed to reverse completely halothane-induced inhibition of [^{3}H]MK-801 binding. It should be noted, however, that even though the anesthetics were less potent at inhibiting [^{3}H]CGS 19755 binding than they were at inhibiting stimulated [^{3}H]MK-801 binding, significant inhibition was, in fact, seen within concentration ranges expected to be achieved during surgical anesthesia (see below). Thus, an effect on glutamate binding is likely to make some contribution to the disruption of NMDA transmission seen during anesthesia.

A third possibility is that anesthetics interact with the glycine binding site. A competitive effect at the glycine site in which glycine and anesthetic binding is mutually exclusive could account for the reversibility of the anesthetic-induced inhibition of glutamate-sensitive [^{3}H]MK-801 binding by glycine. The EC_{50} for glycine potentiation of channel activation by glutamate (as signaled by the increase in [^{3}H]-MK-801 binding) is comparable to the EC_{50} values for glycine reversal of halothane and enflurane inhibition of [^{3}H]MK-801 binding. In contrast, the direct stimulatory effect of glycine on [^{3}H]MK-801 binding, which might involve a low affinity action at the glutamate binding site, required considerably higher concentrations of glycine. That binding inhibition by certain anesthetics such as chloroform and methoxyflurane was less sensitive to reversal by glycine could indicate another site of action by the anesthetics (glutamate binding site). For technical reasons, we were unable to reliably detect the glycine binding site using [^{3}H]glycine as a probe; additional experiments are being undertaken using newer radiolabeled glycine antagonists as specific probes for this site.

There are limitations inherent in using radioligand binding methodology to study the conformational changes associated with the gating of receptor-operated ion channels: receptor channels respond within milliseconds, whereas binding protocols generally encompass extended incubation periods. Thus, it is probable that the binding reflects ligand interactions with a mixed population of resting, activated and desensitized receptors. This raises the possibility that certain of the anesthetic effects involve alterations in the distribution of the receptor between different functional states. Desensitization of NMDA-activated currents has been demonstrated in electrophysiological studies of NMDA receptors in cultured embryonic neurons [24] and expressed in *Xenopus* oocytes [25]. Mayer and coworkers [24] concluded that a major action of glycine on NMDA receptors is to speed up the rate constant of recovery

from desensitization. Lerma and coworkers [25] suggested that desensitization involves an agonist-induced conformational change that results in a reduced affinity for glycine. We recently demonstrated that enflurane and halothane decrease both the rate of onset and the extent of the NMDA-induced desensitization of $^{45}Ca^{2+}$ uptake by rat brain microvesicles [26]. The precise relationship between anesthetic action, desensitization, and the glycine site on the NMDA receptor remains to be established.

It has been reported that under physiological conditions, the glycine receptor is saturated, cerebrospinal fluid glycine concentrations being in the micromolar range [27]. This suggests that volatile anesthetics might have a limited impact on NMDA receptor function *in vivo*. However, the responsiveness of NMDA receptors to glycine antagonists has led to the proposal that local glycine concentrations are maintained low by glycine uptake mechanisms or that endogenous glycine antagonists effectively compete with the glycine receptor [27, 28]. It is also possible that not all NMDA receptor subtypes possess allosteric glycine sites.

Anesthetic potency. Comparing *in vitro* anesthetic concentrations and those that result in clinical anesthesia *in vivo* poses several obstacles. However, the minimal alveolar concentration (MAC) values in rats are 1.04, 2.25, and 1.44% (v/v) for halothane, enflurane, and isoflurane, respectively [29]. Equilibration of the Tris buffers used in the present experiments with 1% (v/v) halothane or 2% (v/v) enflurane at 37° using calibrated, agent-specific vaporizers results in buffer anesthetic concentrations of 0.4 and 0.65 mM [14]. Corresponding isoflurane concentrations are in the range of 0.3 to 0.4 mM. These calculations suggest that the concentrations of anesthetics that affect NMDA receptors are indeed within the range of concentrations achieved in clinical practice. Also, on the basis of MAC values, enflurane would appear to be the most potent of these anesthetics in the inhibition of glutamate-dependent [^{3}H]MK-801 binding.

The anesthetics depressed glutamate stimulation of [^{3}H]MK-801 binding in a concentration-dependent manner with the following order of potency: halothane $\geq$ enflurane $>$ methoxyflurane $>$ chloroform $>$ diethyl ether $>$ isoflurane (Table 1). In terms of their lipophilicity (as indicated by their oil/gas partition coefficients at 37°), the following series is obtained: methoxyflurane $>$ chloroform $=$ halothane $>$ enflurane $=$ isoflurane $>$ diethyl ether (Table 1) [15]. Thus, the strict correlation between lipophilicity and anesthetic potency is not mirrored in the correlation between lipophilicity and NMDA inhibitory potency. The simplest explanation for this discrepancy is that, not surprisingly, the development of the anesthetic state involves neurochemical actions in addition to (or other than) NMDA receptor effects.

Reversibility is a key characteristic of clinically useful anesthetic agents, and is a property expected of the neurochemical actions of anesthetic agents that are, in fact, responsible for producing an anesthetic state. The disruption of glutamate-sensitive channel activation by the anesthetics observed in the present experiments was readily reversed by anesthetic removal from the assay medium.

The present results are consistent with the notion that volatile anesthetics block transmission at NMDA receptors at clinically relevant concentrations and in a manner that is consistent with production of an anesthetic state. This inhibition does not appear to involve direct actions with either the agonist recognition site or the receptor ionophore; rather, the inhibition appears to be specific for the ion channel activation process (i.e. the glutamate-stimulated conformational change that increases the accessibility of [^{3}H]MK-801 for its binding site within the ion channel). It is possible that anesthetic actions at obligatory allosteric sites (e.g. the glycine site) underlies this activity.

Acknowledgements—This work was supported by PHS Grants GM 46408 and NS-25296, and the Research Service of the Veterans Administration.

REFERENCES

1. Foster AC and Fagg GE, Taking apart NMDA receptors. *Nature* **329**: 395–396, 1987.
2. Seeburg PH, The molecular biology of mammalian glutamate receptor channels. *Trends Pharmacol Sci* **14**: 297–303, 1993.
3. Kaplita PV and Ferkany JW, Evidence for direct interactions between NMDA and glycine recognition sites in brain. *Eur J Pharmacol* **188**: 175–179, 1990.
4. Lehmann J, Chapman AG, Meldrum BS, Hutchison A, Tsai C and Wood PL, CGS 19755 is a potent and competitive antagonist at NMDA-type receptors. *Eur J Pharmacol* **154**: 89–93, 1988.
5. Kemp JA, Foster AC and Wong EHF, Non-competitive antagonists of excitatory amino acid receptors. *Trends Neurosci* **10**: 294–298, 1987.
6. Scheller MS, Zornow MH, Fleischer JE, Shearman GT and Greber TF, The noncompetitive *N*-methyl-D-aspartate receptor antagonist, MK-801 profoundly reduces volatile anesthetic requirements in rabbits. *Neuropharmacology* **28**: 677–681, 1989.
7. Daniell LC, The noncompetitive *N*-methyl-D-aspartate antagonists, MK-801, phencyclidine, and ketamine, increase the potency of general anesthetics. *Pharmacol Biochem Behav* **36**: 111–115, 1990.
8. Daniell LC, Effect of CGS 19755, a competitive *N*-methyl-D-aspartate antagonist, on general anesthetic potency. *Pharmacol Biochem Behav* **40**: 767–769, 1991.
9. Daniell LC, Alteration of general anesthetic potency by agonists and antagonists of the polyamine binding site of the *N*-methyl-D-aspartate receptor. *J Pharmacol Exp Ther* **261**: 304–310, 1992.
10. Yang J and Zorumski CF, Effects of isoflurane on *N*-methyl-D-aspartate gated ion channels in cultured rat hippocampal neurons. *Ann NY Acad Sci* **625**: 287–289, 1991.
11. Puil E, El-Beheiry H and Baimbridge KG, Anesthetic effects on glutamate-stimulated increase in intraneuronal calcium. *J Pharmacol Exp Ther* **255**: 955–961, 1990.
12. Martin DC, Abraham JE, Plagenhoef M and Aronstam RS, Volatile anesthetics and NMDA receptors. Enflurane inhibition of glutamate-stimulated [^{3}H]MK-801 binding and reversal by glycine. *Neurosci Lett* **132**: 73–76, 1991.
13. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenck DC, Measurement of

protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
14. Martin DC, Pruett JK and Aronstam RS, Test-tube equilibration of volatile anaesthetic concentrations. *Br J Anaesthesia* **70**: 365–367, 1993.
15. Koblin DD and Eger EI, How do inhaled anesthetics work? In: *Anesthesia* (Ed. Miller RD), 2nd Edn, pp. 581–623. Churchill Livingstone, New York, 1986.
16. Yamamura T, Harada K, Okamura A and Kemmotsu O, Is the site of action of ketamine anesthesia the *N*-methyl-D-aspartate receptor? *Anesthesiology* **72**: 704–710, 1990.
17. Davies SN, Alford ST, Coan EJ, Lester RAJ and Collinridge GL, Ketamine blocks an NMDA receptor-mediated component of synaptic transmission in rat hippocampus in a voltage-dependent manner. *Neurosci Lett* **92**: 213–217, 1988.
18. Rabe CS and Tabakoff B, Glycine site-directed agonists reverse the actions of ethanol at the *N*-methyl-D-aspartate receptor. *Mol Pharmacol* **38**: 753–757, 1990.
19. Martin DC, Dennison RL and Aronstam RS, Barbiturate interactions with *N*-methyl-D-aspartate (NMDA) receptors in rat brain. *Mol Neuropharmacol* **2**: 255–259, 1992.
20. Teichberg VI, Tal N, Goldberg O and Luini A, Barbiturates, alcohols and CNS excitatory neurotransmission: Specific effects on the kainate and quisqualate receptors. *Brain Res* **291**: 285–292, 1984.
21. Huettner JE and Bean BP, Block of *N*-methyl-D-aspartate-activated current by the anticonvulsant MK-801: Selective binding to open channels. *Proc Natl Acad Sci USA* **85**: 1307–1311, 1988.
22. Foster AC and Wong EHF, The novel anticonvulsant MK-801 binds to the activated state of the *N*-methyl-D-aspartate receptor in rat brain. *Br J Pharmacol* **91**: 403–409, 1987.
23. Bonhaus DW and McNamara JO, Uncompetitive antagonist binding: A biochemical index of activation of the NMDA receptor-coupled ion channel. *Epilepsy Res Suppl* **8**: 181–188, 1992.
24. Mayer ML, Vyklicky L and Clements J, Regulation of NMDA receptor desensitization in mouse hippocampal neurons by glycine. *Nature* **33**: 425–427, 1989.
25. Lerma J, Zukin RS and Bennett MVL, Glycine decreases desensitization of *N*-methyl-D-aspartate (NMDA) receptors expressed in *Xenopus* oocytes and is required for NMDA responses. *Proc Natl Acad Sci USA* **87**: 2354–2358, 1990.
26. Aronstam RS, Martin DC and Dennison RL, Volatile anesthetics inhibit NMDA-stimulated ^{45}Ca uptake by rat brain microvesicles. *Neurochem Res*, in press.
27. Huettner JE, Competitive antagonism of glycine at the *N*-methyl-D-aspartate (NMDA) receptor. *Biochem Pharmacol* **41**: 9–16, 1991.
28. Thomson AM, Glycine modulation of the NMDA receptor/channel complex. *Trends Neurosci* **12**: 349–353, 1989.
29. Mazze RI, Rice SA and Baden JM, Halothane, isoflurane, and enflurane MAC in pregnant and nonpregnant female and male mice and rats. *Anesthesiology* **62**: 339–341, 1985.