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Saturable binding of halothane to rat brain synaptosomes

(anesthetic/ethanol/inhalational anesthetics/photolysis/photoaffinity)

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ABSTRACT **The hypothesis that volatile anesthetics act directly on or bind specifically to membrane proteins remains controversial. In earlier *in situ* electron probe microanalysis studies in cardiac muscle we showed preferential partitioning of halothane into mitochondria. To determine whether partitioning represents saturable binding or simple solubility, a photoaffinity labeling method was developed for halothane to examine binding in rat brain synaptosomes. Radioligand binding assays were then used to determine binding parameters for this important inhalational anesthetic. UV-light exposure of synaptosomes incubated with clinical concentrations of [^{14}C]halothane resulted in sufficient labeling to allow characterization of binding sites. Analysis of saturation and competition curves showed that >60% of [^{14}C]halothane photolysis product binding to synaptosomes was specific, with low affinity (K_d = 0.49 ± 0.16 mM) and high binding site concentration (B_{max} = 1.87 ± 0.75 nmol/mg of protein). Halothane photoaffinity labeling was partially inhibited by isoflurane (20%), chloroform (44%), 2-bromotrifluoroethane (20%), and dichlorotrifluoroethane (20%) but not by ethanol. The K_d measured with this photoaffinity approach is similar to the concentration of halothane required to produce anesthesia in rats.**

The delivery and use of inhalational anesthetics have reached a highly sophisticated level; however, an understanding of how and where they produce their various effects remains elusive (1–3). Historically, three principal methods have been used to probe for sites and mechanisms of action of drugs: analysis of structure–activity relationships, functional studies, and radioligand binding assays. Structure–activity studies of anesthetics, as characterized by the Meyer–Overton relationship (4), have broadly defined the molecular target to be hydrophobic, but they have not provided evidence distinguishing between a direct lipid or protein site of action. Functional studies, on the other hand, seem to point to anesthetic effect at multiple protein sites (5–7). However, because the function of many proteins depends on membrane lipid, determination of the direct anesthetic site remains ambiguous. Radioactive ligand binding techniques, powerful methods for identifying and characterizing pharmacological sites of action, have not previously been used to study the inhalational anesthetics, primarily because of their low affinity, rapid kinetics, high vapor pressure, and absence of chemical antagonists. We have previously shown that the *in situ* halothane distribution could be fixed in place by exposure to the high-energy electron beam of a electron microscope (8). After this radiolysis, subcellular halothane concentration was determined with x-ray energy-dispersive microanalysis of the element bromine. We found significant heterogenous partitioning, with highest levels in mitochondria. To dissect this partitioning further and begin a systematic search for sites of anesthetic action at the molecular level, we have developed a photoaffinity labeling technique that uses radiolabeled halothane as the ligand.

Photoaffinity labeling is a method that allows irreversible covalent labeling of normally reversible ligand binding sites by the conversion of the stable ligand into highly reactive species with UV light (9). Although many photoaffinity ligands are chemically modified to incorporate a photolabile group, unmodified halothane readily decomposes in 254-nm UV light to bromine and trifluorochloroethane free radicals (10). The highly reactive ethane radicals are expected to bind rapidly and covalently, near to their reversible pharmacologic binding sites (11). Characterization of such labeled sites can then proceed with conventional methods.

MATERIALS AND METHODS

Tissue Preparation. Animal protocols were approved by the Institutional Animal Care and Use Committee. The whole brains (≈2 g) of male adult Sprague–Dawley rats were removed immediately on achieving a satisfactory anesthetic state (50 mg of pentobarbital per kg i.p.), washed, minced, and homogenized in 10 volumes of ice-cold 1.0 mM Na_2HPO_4/0.32 M sucrose buffer, pH 7.4, using a Potter–Elevehjem glass homogenizer with Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min, and the pellet was rehomogenized in half the original volume of buffer and recentrifuged at the same force. The supernatant fractions from the first and second separations were combined and centrifuged at 30,000 × *g* for 30 min. The resultant pellet containing synaptosomal membranes was suspended in 10 volumes of sucrose phosphate buffer and used immediately for binding assays. Protein concentration was determined by Coomassie blue assay (12).

Binding Assay. [^{14}C]Halothane, 0.83 mCi/mmol (New England Nuclear; 1 Ci = 37 GBq), was diluted 1:1 in ethanol over dry ice, dissolved in 1 mM Na_2HPO_4/0.32 M sucrose buffer, pH 7.4, to a final concentration of 4 mM, and stored at −80°C. This was done immediately on receipt to reduce the possibility of β degradation. Photolysis samples were prepared in triplicate as follows: aliquots (0.2 ml) of synaptosomes were added to 5-mm pathlength Suprasil cuvettes (2-ml volume; Markson, Phoenix, AZ). Glutathione was added to a final concentration of 10 mM as a scavenger for aqueous phase radicals (13), and [^{14}C]halothane, dissolved in buffer as above, was injected from a repeating gas-tight Hamilton syringe into the mixture. Volumes were adjusted so that the final addition completely filled the cuvette, which was then sealed with a Teflon stopper and incubated for 1 min with stirring. The mixture was then exposed to the focused UV output of a 200-W HBO bulb after $CuSO_4$ solution IR filtering and reflection from a dichroic mirror (<320 nm reflected; Oriel, Stratford, CT) with constant stirring for various lengths of time (see below). All of these steps were

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performed at 4°C. Bound label was separated from free by vacuum filtration over Whatman GF/B glass fiber filters pretreated with 0.05% polyethylenimine (Sigma) and washed with 10 ml of 2°C buffer. The filters were added to scintillation fluid and assayed at 95% efficiency in a Wallace 1209 scintillation counter.

Light/Dark Experiments. Samples were prepared as above, and halothane was added to a final concentration of 0.08 mM and exposed for various periods of time (0, 1, 2, 5, 10 min) to UV light or to ambient room (fluorescent) light with constant stirring.

Saturation Experiments. Two sets of samples were prepared. In the first set, [^{14}C]halothane, in an increasing concentration from 0.2 mM to 2.0 mM, was incubated with a constant concentration of synaptosomal protein and exposed to UV light for 10 sec; bound label was determined as a function of [^{14}C]halothane concentration. In the second set of samples 2.5 mM unlabeled halothane was added to the reaction mixture of each [^{14}C]halothane concentration and bound label was again determined after photolysis. This was considered the nondisplaceable (or nonspecific) binding for subtraction from the total binding curve to produce the saturation binding isotherm. Conventional determination of nonspecific binding uses an unlabeled ligand concentration in a 1000-fold excess over the labeled ligand. This was not possible for halothane since it would exceed halothane's solubility in buffer. Furthermore, halothane concentrations in excess of 7 mM have been reported to extract specific proteins from mitochondria (14) and may also solubilize lipid, both of which may alter binding parameters.

Competition Experiments. Synaptosomes were incubated with 0.2 mM [^{14}C]halothane, 10 mM glutathione, and various concentrations of unlabeled anesthetics: halothane (Halocarbon Laboratories, Hackensack, NJ), isoflurane (Anaquest, Murray Hill, NJ), chloroform (Sigma), bromotrifluoroethane (Aldrich), dichlorotrifluoroethane (Aldrich), or ethanol. The mixtures were then exposed to UV light for 10 sec with constant stirring as above. The radioactivity retained on filter was determined by liquid scintillation spectroscopy.

Data Analysis. All assays were performed in triplicate and values obtained from three separate experiments were used to calculate means and standard errors. Iterative nonlinear regression analysis of binding data was performed with the EBDA (equilibrium binding data analysis) (15) program for an IBM PC.

RESULTS

Light/Dark and Time Course Experiments. Because labeling of synaptosomal tissue may occur in the absence of UV light, due either to metabolic activation (16, 17) or to contamination of the labeled halothane stock with reactive halothane degradative products (17), identically prepared samples were exposed to either ambient or UV light for identical time courses. Total label incorporation in synapatosomes increased nearly linearly (Fig. 1) with increasing UV-exposure. However, in ambient light, there was no apparent increase in label incorporation over the 10-min exposure. At 10 min, UV exposure resulted in a 140-fold higher ^{14}C incorporation over ambient light. In other experiments (data not shown) complete dark conditions resulted in ^{14}C incorporation that was not significantly different from the ambient light values shown in Fig. 1.

Saturation Experiments. Scatchard analysis of saturation curves allows calculation of binding site density (B_{max}), dissociation constant (K_d), and Hill coefficient. The results of these studies are shown in Fig. 2, before and after subtraction of nonspecific binding. Binding parameters determined from iterative computer analysis (EBDA) for this isotherm were K_d = 0.49 ± 0.16 mM, B_{max} = 1.87 ± 0.75 nmol/mg of protein,

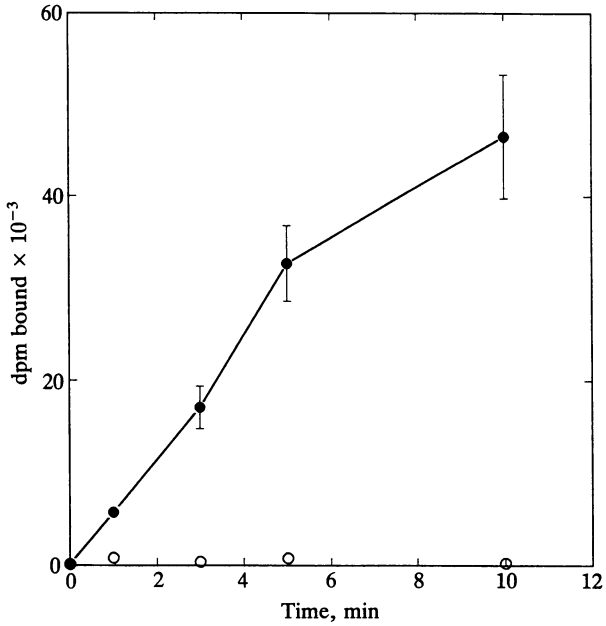

FIG. 1. Effect of light on [^{14}C]halothane (0.08 mM) binding to synaptosomal membranes. Retained radioactivity was measured after incubation at 22°C in ambient light (○) and with UV light (●) for various times. Protein concentration was 1.60 mg per assay and specific activity was 1.735 cpm/pmol.

and Hill coefficient n = 0.92 ± 0.02. As anticipated from an inspection of Fig. 1, B_{max} is dependent on duration of UV exposure, but preliminary studies (data not shown) demonstrated that K_d and the Hill coefficient were not.

Competition Experiments. To determine the specificity of halothane photoaffinity binding sites in rat synaptosomes, the ability of unlabeled halothane and other anesthetics to compete with labeled photolysis product binding was studied. The apparent specificity and affinity of [^{14}C]halothane photolysis product binding were demonstrated in the competition assay by the ability of unlabeled halothane (Fig. 3) to reduce incorporated label by 63% in synaptosomes. Iterative nonlinear regression analysis (15) of the competition curves gave the binding parameters of K_d = 0.52 ± 0.21 mM and B_{max} = 2.1 ± 0.75 nmol/mg of protein, with a Hill coefficient of n = 0.71 ± 0.33, similar to the saturation values. Chloroform inhibited binding by about 44%, whereas isoflurane, dichlorotrifluoroethane, and 2-bromotrifluoroethane reduced incorporated label by only 20%. On the other hand, ethanol, a hydrophobic-phase radical scavenger, was unable to reduce ^{14}C incorporation in rat brain synaptosomes (Fig. 4).

DISCUSSION

Halothane Binding Site. The binding assay approach to the characterization of halothane or other inhalational anesthetic binding sites has been neglected because of the low affinity of any apparent binding and the high volatility of the agents. This approach has also been unattractive because of difficulties in conceiving of a common and specific functional site that could accommodate the large variety of structures capable of producing anesthesia. Nevertheless, recent studies have suggested that the halocarbon anesthetics could produce their effect by a direct interaction with protein (18), as opposed to an indirect and nonspecific effect on membrane lipid. Using photoaffinity labeling, this study suggests displaceable binding within the nontoxic concentration range. The simplest explanation for these results is that saturable binding represents a specific halothane site on protein, and nonsaturable binding represents lipid solubility or nonspe-

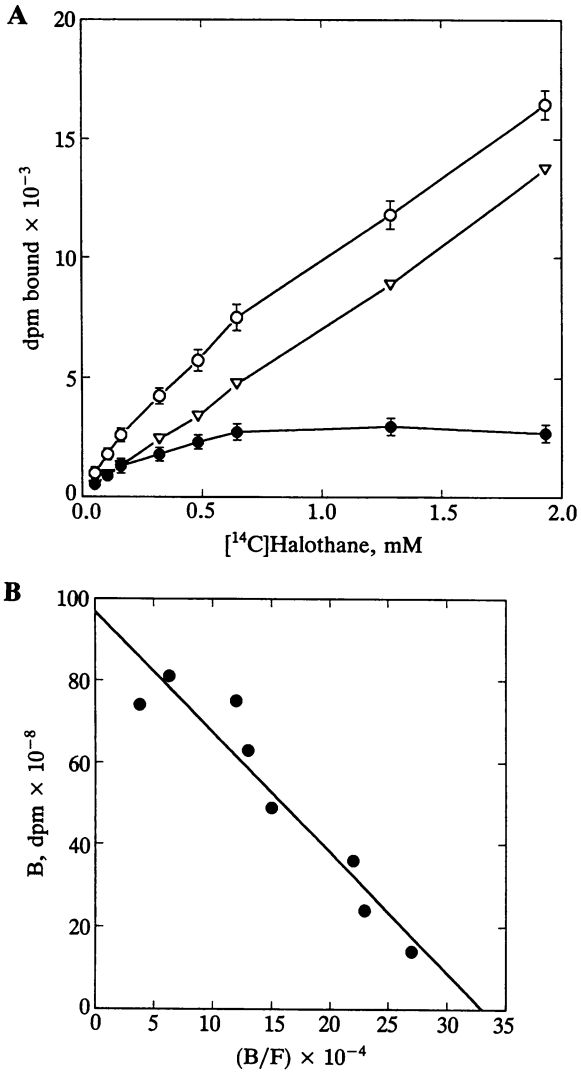

FIG. 2. Scatchard analysis of halothane binding. (*A*) Saturation isotherm before (○) and after (●) subtracting nonspecific binding (▽), as determined by adding 2.5 mM unlabeled halothane in the competition experiment. Results are expressed as mean ± SEM for three experiments. (*B*) Scatchard plot of data after removal of nonspecific binding. B, bound; F, free.

cific binding. Recent fluorine NMR experiments have also described two chemical environments for halothane, one being more restricted than the other (19); however, neither NMR site showed saturation within the clinical concentration range (20). The use of whole brain for these NMR experiments may have decreased the concentration of saturable sites to below detectable limits for the NMR technique. Although saturable binding implies that the site is protein, a lipid site cannot be excluded, since saturable anesthetic binding at high concentrations has been observed at a lipid water interface (21). However, even if protein, the saturable binding site identified in this study is unlikely to be a classic receptor because of its low affinity (high K_d) and high binding site density (B_{max}). These results are most consistent with weak binding to many sites, such as the hydrophobic transmembrane sequences common to many receptors, channels, and ion pumps. The similarity of K_d to ED_{50} for this anesthetic suggests that the saturable site has relevance to mechanisms of anesthesia, a possibility that can be examined when the site or sites have been identified. Finally, the inability of other, similar anesthetics, all at clinically effective concentrations, to significantly inhibit halothane photolysis product binding suggests partial displacement and a number of separate or overlapping sites may exist on the same or different proteins. This may, in part, explain the marked differences in action between the different volatile agents.

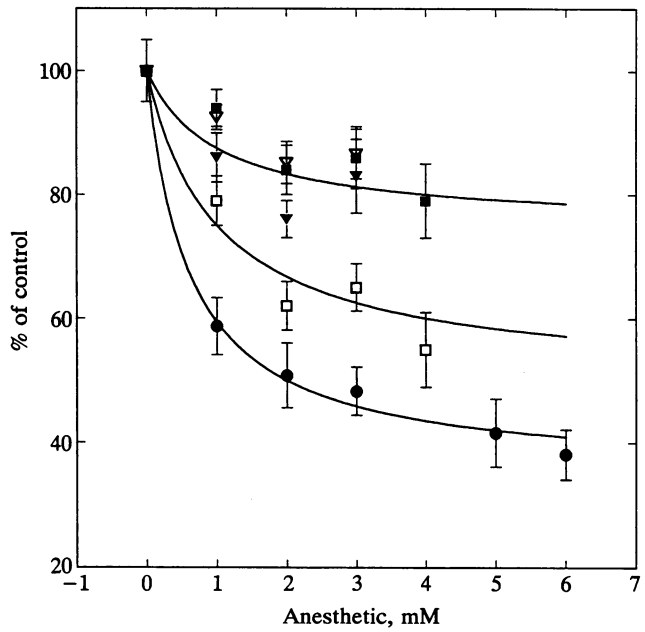

FIG. 3. Inhibition of halothane binding by various inhalational anesthetics: halothane (●), isoflurane (▼), chloroform (□), dichlorotrifluroethane (■), and 2-bromotrifluroethane (▽). Specificity of [^{14}C]halothane is displayed by the ability of unlabeled halothane to displace labeled halothane (63%) and for isoflurane, chloroform, bromotrifluroethane, and dichlorotrifluroethane to partially displace labeled halothane. Solid lines are competitive inhibition curves fit by eye, assuming incomplete overlap of anesthetic binding sites. Results are expressed as means ± SEM for three experiments.

Halothane Photoaffinity Labeling. Photoaffinity labeling has been used to identify the binding sites of many ligands on a molecular and submolecular level (22). Using halothane as a photoaffinity ligand, we have developed a technique that allows the use of classic pharmacological methodology to study binding of anesthetics at a subcellular level. We have been able to use this approach because (*i*) halothane is photolabile without chemical modification and (*ii*) covalent linkage of the halothane photolysis product to tissue allows the use of vacuum filtration to separate bound from unbound label. Total halothane bound to synaptosomes after 10 sec of UV exposure at ED_{50} buffer concentrations (0.34 mM, 4°C)

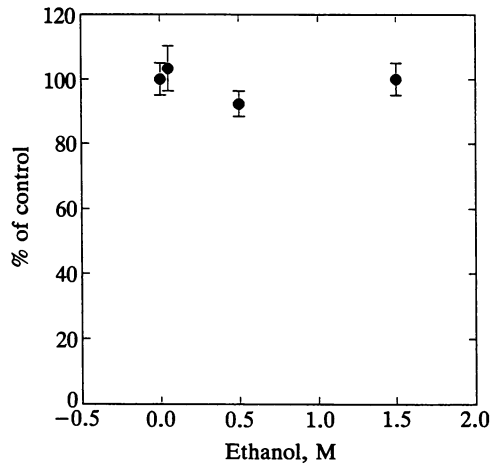

FIG. 4. Effect of ethanol on [^{14}C]halothane binding to brain synaptosomal membranes. Results are expressed as means ± SEM for three experiments.

and is calculated to be about 1.6 nmol/mg of protein. This value cannot be used to calculate partition coefficients (23), however, because of the time-dependent increase in label incorporation (Fig. 1). The UV-exposure duration necessary to produce "physiologic" partitioning is not clear since halothane partitioning may be altered by simultaneous and cumulative tissue photolytic damage.

The photolysis and covalent linkage of halothane is rapid; only a 10-sec exposure with 254-nm UV light from a 200-W Hg lamp is required for 5% covalent linkage (Fig. 1). The time dependence of covalent linkage could be adequately described by a single exponential or pseudo-first-order kinetics suggesting a one-step linear process requiring either the formation of a target free radical or, more likely, a single halothane free radical. For halothane to reliably label sites of reversible pharmacologic binding, it is necessary that the covalent bond between the photoactivated halothane and tissue site form faster than the normal halothane off-rate (assuming that the off-rate for the halothane photolysis product is the same as for halothane). The reversibility of halothane binding is demonstrated by the very low retained radioactivity after exposure to ambient light (Fig. 1). Estimations of the halothane off-rates are possible based on our measured K_d and assuming that the binding rate ($k_+ = 10^8$ liter/mol · sec) is diffusion-limited. The halothane dissociation rate constant ($k_{-1} = k_{+1} \cdot K_d$) estimated in this way is 6.25×10^4 sec^{-1}, consistent with the estimate of $3\text{–}5 \times 10^3$ sec^{-1} made for halothane bound to albumin using fluorine NMR (A. S. Evers, personal communication). Although we have not directly measured the reaction rate of activated halothane with tissue sites, it is likely to be several orders of magnitude faster, especially if halothane is in a restricted, or "caged," protein environment when activated (24). Thus, it is probable that sites of reversible halothane binding will be reliably labeled, instead of ethane free radicals diffusing to sites of preferential reaction (such as double bonds). This is confirmed by the inability of ethanol to alter halothane binding, since ethanol should be able to scavenge ethane radicals diffusing in the hydrophobic phase. Thus, we believe that the photoaffinity approach results in reliable labeling of tissue sites normally occupied by halothane under physiologic conditions.

Alternative Interpretations. At least three other possibilities could explain the photoaffinity results apart from a specific binding site. (*i*) Since halothane binding sites are likely to be hydrophobic, it is possible that high concentrations of halothane may actually elute those sites so that bound label is soluble and passes through the filter. Analysis of the filtrate showed only a 3% increase in protein concentration with the highest halothane concentrations used in this study, in agreement with published data (14). In addition, the inability of other organic solvents, such as chloroform or ethanol, to displace labeled halothane makes this possibility unlikely.

(*ii*) Halothane photolysis products may react with each other or with other halothane molecules as the concentration increases in particularly soluble environments. Thus, instead of [^{14}C]halothane covalently binding to tissue, the label would remain soluble and not be retained on the filters. This would be more probable as the concentration of halothane is increased, mimicking displacement or saturation curves. If true, halothane dimerization should result, but in preliminary studies using gas chromatography we were unable to find any evidence for the most likely dimer, dichlorohexafluorobutene (25). In addition, ethanol, a hydrophobic-phase radical scavenger, was unable to inhibit halothane photolysis product binding. Taken together, these results suggest that this "self-quenching" is not responsible for the inhibition of binding observed with unlabeled halothane.

(*iii*) Increasing buffer concentrations of halothane may absorb sufficient UV light to decrease the efficiency of photolysis. We measured the UV absorbance spectra of 5 mM halothane in buffer and found that the extinction length at 254 nm was 20 cm, or 40 times the cuvette pathlength. This result makes excess UV absorption an unlikely explanation for the apparent inhibition of binding.

Conclusion. Using a photoaffinity modification of the radioligand binding assay, we have identified a saturable binding site in rat brain tissue for a halothane photolysis product, which we believe indicates that a saturable environment exists for the clinically important inhalational anesthetic halothane. The apparent affinity of this site is consistent with the halothane concentrations required for an anesthetic effect.

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