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Pharmacology

Volatile anesthetics compete for common binding sites on bovine serum albumin: A ^{19}F-NMR study

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ABSTRACT **There is controversy as to the molecular nature of volatile anesthetic target sites. One proposal is that volatile anesthetics bind directly to hydrophobic binding sites on certain sensitive target proteins. Consistent with this hypothesis, we have previously shown that a fluorinated volatile anesthetic, isoflurane, binds saturably [K_d (dissociation constant) = 1.4 ± 0.2 mM, B_{max} = 4.2 ± 0.3 sites] to fatty acid-displaceable domains on serum albumin. In the current study, we used ^{19}F-NMR T_2 relaxation to examine whether other volatile anesthetics bind to the same sites on albumin and, if so, whether they vary in their affinity for these sites. We show that three other fluorinated volatile anesthetics bind with varying affinity to fatty acid-displaceable domains on serum albumin: halothane, K_d = 1.3 ± 0.2 mM; methoxyflurane, K_d = 2.6 ± 0.3 mM; and sevoflurane, K_d = 4.5 ± 0.6 mM. These three anesthetics inhibit isoflurane binding in a competitive manner: halothane, K_i (inhibition constant) = 1.3 ± 0.2 mM; methoxyflurane, K_i = 2.5 ± 0.4 mM; and sevoflurane, K_i = 5.4 ± 0.7 mM—similar to each anesthetic's respective K_d of binding to fatty acid displaceable sites. These results illustrate that a variety of volatile anesthetics can compete for binding to specific sites on a protein.**

Volatile anesthetics have been traditionally viewed as "nonspecific" drugs that act by perturbing the structure of lipid membranes and secondarily affecting the function of membrane proteins. There is, however, accumulating evidence that these drugs act by direct binding to protein target sites (1, 2). Action by direct binding is suggested by volatile anesthetic inhibition of the water-soluble enzyme firefly luciferase (1, 3) as well as by the stereoselective modulation of certain neuronal ion channels by optical isomers of isoflurane (CHF_2—O—CHCl—CF_3) (4).

Recently, ^{19}F-NMR spectroscopy was utilized to directly demonstrate and characterize the binding of a fluorinated volatile anesthetic, isoflurane, to bovine serum albumin (BSA) (5). Saturable binding was observed (dissociation constant K_d = 1.4 ± 0.2 mM) and was shown to be displaceable by oleic acid, a long-chain fatty acid and high-affinity ligand for albumin. When oleic acid was present at a 6:1 mol ratio with BSA, isoflurane binding was reduced to a residual, nonsaturable binding component. The fatty acid-displaceable isoflurane binding was quantified and corroborated by a gas chromatographic partition analysis (B_{max} = 4.2 ± 0.3 sites; K_d = 1.4 mM ± 0.2 mM). This demonstration was a first step in characterizing specific domains on proteins that saturably bind volatile anesthetics.

In the current study, we utilized BSA as a model system to investigate several fundamental questions about anesthetic pharmacology. First, it is unclear whether all volatile anesthetics bind to similar target site(s) (6) and, if so, whether they bind to these sites with different affinities. This is of interest because differences in affinity for protein target sites could provide a basis for observed differences in *in vivo* anesthetic potency. To address this question, the binding of several fluorinated anesthetics, halothane (CF_3—CHClBr), methoxyflurane (CH_3—O—CF_2—$CHCl_2$), and sevoflurane ((CF_3)$_2$CH—O—CH_2F), to fatty acid-displaceable sites on BSA was characterized by using ^{19}F-NMR relaxation. Second, if volatile anesthetics do act at a common site, they should compete for binding at that site. Whereas volatile anesthetics have been shown to produce additive behavioral effects (7), competitive binding of volatile anesthetics has not been demonstrated in any system. Again by using ^{19}F-NMR, the three fluorinated anesthetics and 1-octanol were tested for their ability to displace isoflurane binding to saturable sites on BSA. It is demonstrated that each anesthetic inhibits isoflurane binding with an inhibition constant, K_i, similar to its K_d of binding to fatty acid-displaceable sites on BSA. These data indicate that several volatile anesthetics compete for binding to specific sites on a model protein.

MATERIALS AND METHODS

Materials. Lyophilized BSA (fraction V, fatty-acid free; purity > 96% by electrophoresis) was purchased from Calbiochem. Sodium oleate was purchased from Sigma. Isoflurane and halothane, both purchased as a racemic mixture of isomers, were obtained from Anaquest (Madison, WI) and Ayerst Laboratories. Methoxyflurane was purchased from Abbott, and sevoflurane was provided by Maruishi Pharmaceuticals (Osaka).

Methods. *Preparation of albumin solutions.* Lyophilized BSA was dissolved by gentle magnetic stirring into a 5 mM sodium Hepes buffer (pH = 7.2) containing 135 mM KCl, 15 mM sodium gluconate, 1 mM EDTA, 0.75 mM $CaCl_2$, and 10 mM glucose. ^{19}F-NMR T_2 measurements were performed with solutions of BSA (M_r = 66,200) at 1.75 mg/ml (26.4 μM) or 1.5 mg/ml (22.6 μM).

Preparation of fatty acid–albumin solutions. Oleic acid was complexed to BSA as described (5). Molar ratios ranging from 3.0 to 12.0 were prepared.

^{19}F-NMR experiments. NMR samples (2 ml) were equilibrated in gas-sampling bulbs (1 liter) with known concentrations of volatilized anesthetic. An additional volatilized anesthetic was also present in the competition studies. After equilibration, a gas-tight syringe was used to transfer samples into 5-mm NMR tubes, which were capped immediately.

Aqueous anesthetic concentrations were measured by equilibrating buffer samples in parallel fashion and determining the anesthetic concentrations of the buffer samples. Anesthetic concentrations were determined by extraction into heptane followed by capillary gas chromatography (8).

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Abbreviations: BSA, bovine serum albumin; CPMG, Carr–Purcell–Meiboom–Gill.

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^{19}F-NMR measurements were obtained on a Varian VXR-500 multinuclear spectrometer operating at 470.3 MHz. Experiments were temperature-controlled at 22°C, with ^2H_2O serving as both a lock and shim signal. Spin–spin relaxation (T_2) measurements were obtained as described (5) by using the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence.

Equilibrium binding constant, K_d, by ^{19}F-NMR T_2 analysis. The dependence of T_2 on anesthetic concentration was used to characterize anesthetic binding. A fast CPMG pulsing rate was used in measuring the relaxation rate ($1/T_2$). The fast CPMG pulsing rate effected a spin-lock, eliminating chemical-shift contributions to the measured relaxation rate. In all experiments, the anesthetic ligand concentration (0.5–5.5 mM) was present in large excess over the BSA concentration (26 μM). The ligand fraction bound, X_b was therefore small, and the relaxation rate ($1/T_2$) of the ligand is given (9, 11)

$$1/T_2 = 1/T_{2f} + X_b/(T_{2b} + \tau_b) \quad [1]$$

where $1/T_{2f}$ and $1/T_{2b}$ are the individual relaxation rates of the free and bound ligand, and τ_b is the lifetime of the bound anesthetic molecules.

A simple bimolecular binding process is illustrated:

$$[A] + [R] \rightleftharpoons [RA], \quad [2]$$

where [A] is the free aqueous anesthetic concentration, which approximates the total anesthetic concentration;† [R] is the total displaceable binding site concentration; and [RA] is the receptor complex concentration.

By defining

$$K_d = [R][A]/[RA] \quad \text{and} \quad X_b \approx [RA]/[A],$$

where K_d is the equilibrium binding (dissociation) constant, Eq. 1 by substitution takes the form (12–14).

$$T_{2p} = [A](T_{2b} + \tau_b)/[R] + K_d(T_{2b} + \tau_b)/[R] \quad [3]$$

where $T_{2p} = (1/T_2 - 1/T_{2f})^{-1}$.

For each anesthetic, saturable binding is shown to be eliminated by adding oleic acid, a high-affinity ligand for albumin. To isolate the K_d of the saturable binding component, the T_2 observed in the presence of oleic acid (T_{2oleic}), is substituted for T_{2f} in the definition of T_{2p}. An X–Y plot of T_{2p} versus the anesthetic concentration, [A], is linear with a slope $= (T_{2b} + \tau_b)/[R]$, and an x-intercept $= -K_d$.

For isoflurane, the total oleic acid-displaceable binding site concentration, [R], was determined by multiplying the total number of displaceable binding sites per BSA molecule, which equaled 4.2 by gas chromatographic partition analysis (5), by the BSA concentration (μM). This value enabled a determination of $(T_{2b} + \tau_b)$ from the slope.

Anesthetic K_i by ^{19}F-NMR T_2 analysis. The ability of other volatile anesthetics to compete with isoflurane for oleic acid-displaceable binding was determined. It should be noted that the trifluoro- or difluoromethylene chemical shift of each competing anesthetic was well separated from the trifluoromethyl group of isoflurane. In principle, increasing concentrations of a competitive anesthetic should cause the T_2 of isoflurane to approach T_{2oleic}. Under such competitive conditions, the relaxation rate of (isoflurane) as a function of the aqueous concentration of the competing ligand, [I], is given by (12)

$$T_{2p} = [I](K_d/K_i)(T_{2b} + \tau_b)/[R] + (T_{2b} + \tau_b)(K_d + [A])/[R], \quad [4]$$

where K_i is the inhibition constant of the competing ligand. A plot of T_{2p} versus [I] has a slope $= (K_d/K_i)(T_{2b} + \tau_b)/[R]$. The slope was used to calculate the K_i of the competing anesthetic. T_{2oleic} was measured in the presence of the various competing anesthetic concentrations. These values were almost identical to the original T_{2oleic} but were used in calculating T_{2p} to offset any reduction in non-oleic-acid-displaceable isoflurane binding. T_{2p} was calculated ($1/T_{2p} = 1/T_2 - 1/T_{2oleic}$).

RESULTS

Oleic Acid Inhibits Saturable Binding of Volatile Anesthetics to Albumin. ^{19}F-NMR was used to characterize the binding of methoxyflurane, halothane, and sevoflurane to BSA and to determine if these anesthetics also bind to fatty acid-displaceable sites. The trifluoromethyl group was observed for halothane and sevoflurane, while the difluoromethylene group of methoxyflurane was observed. A single trifluoromethyl or difluoromethylene resonance was always observed in aqueous BSA, with a chemical shift that varied as a function of anesthetic and BSA concentration (5). This indicated that the anesthetic ligand was rapidly exchanging between an aqueous (free) environment and a chemically shifted, protein-bound environment. To measure T_2, a CPMG 180° interpulse interval of 100 μsec was found to be sufficiently short to negate any contribution of the chemical-shift difference between the free and bound ligand (5). The T_2 relaxation for each anesthetic, in buffer and in BSA solution, was strictly uniexponential.

In buffer, the T_2 of halothane, methoxyflurane, and sevoflurane equaled 890 ± 20, 592 ± 18, and 2301 ± 45 msec ± SD, respectively, and showed no dependence on anesthetic concentration. In BSA solution (1.75 mg/ml), the T_2 was much shorter and showed a strong dependence on anesthetic concentration (Fig. 1*A*). The trifluoromethyl T_2 of isoflurane changed analogously in BSA solution (1.75 mg/ml) (5) and is plotted for comparison (Fig. 1*A*). The increase in T_2 was expected with saturable anesthetic binding because X_b, the anesthetic fraction bound, decreased with increasing buffer anesthetic concentration as the limited number of saturable sites became occupied.

When oleic acid (6:1 mole ratio) was added to the BSA solutions, the T_2 dramatically increased for each of the anesthetics, and the T_2 dependence on anesthetic concentration was abolished (Fig. 1*A*). The results were entirely analogous to the case of isoflurane. The increase in isoflurane's T_2 with the addition of oleic acid was previously shown to be correlated with a decrease in binding to BSA, as shown by gas chromatographic partition analysis. Similarly, the lack of a T_2 dependence on anesthetic concentration was shown to be correlated with a loss of saturable, higher affinity binding.

The concentration-dependent inhibition of anesthetic binding by oleic acid is illustrated in Fig. 1*B*. The relaxation rate, $1/T_2$, of each anesthetic (at 2.0 mM) in BSA solution (1.5 mg/ml) is plotted as a function of the mole ratio of oleic acid/BSA. In each case, T_2 approximately doubled with the addition of oleic acid. The oleic acid stoichiometric requirement for displacement of anesthetic binding was indistinguishable for each anesthetic; in all cases a 6:1 oleic acid/BSA mole ratio maximally inhibited anesthetic binding.

Inhibition of saturable volatile anesthetic binding was also achieved by lowering the solution pH to 2.5 (not shown). For each anesthetic, the T_2 increased to a level comparable to that observed with oleic acid, and the T_2 dependence on anesthetic concentration was abolished. The pH-induced confor-

†In all experiments, [A], the free aqueous anesthetic concentration, was achieved by equilibration with a functionally infinite gas reservoir. Consequently, protein binding does not alter [A] in the liquid sample. In the experiments with a competing volatile anesthetic [I], the aqueous concentration of competitor is similarly unaffected by binding.

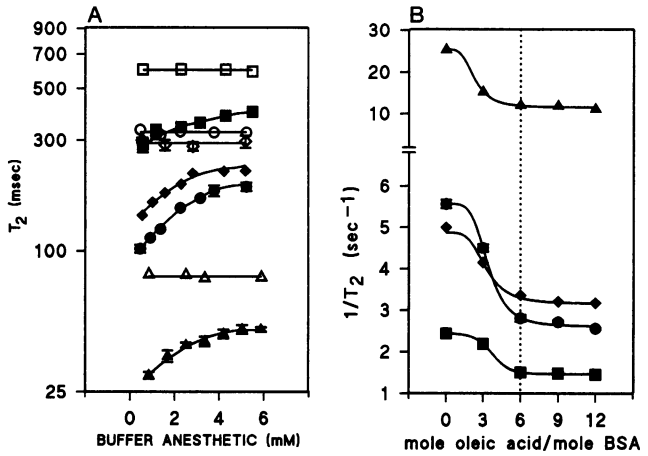

FIG. 1. (*A*) Observed ^{19}F-NMR trifluoromethyl T_2 of halothane (◆), sevoflurane (■), and isoflurane (●) in fatty acid-free BSA (1.75 mg/ml) and in oleic acid-complexed BSA (1.75 mg/ml) (6:1 oleic acid/BSA mole ratio) (◇, □, ○) solutions. Also plotted is the ^{19}F-NMR difluoromethylene T_2 of methoxyflurane in fatty acid-free BSA (1.75 mg/ml) (▲) and in oleic acid-complexed BSA (1.75 mg/ml) (6:1 oleic acid/BSA mole ratio) (△) (mean ± SD). (*B*) Observed ^{19}F-NMR trifluoromethyl ($1/T_2$) of halothane (◆), sevoflurane (■), and isoflurane (●) at 2.0 mM aqueous (free) anesthetic concentration in BSA solution (1.5 mg/ml), plotted as a function of the oleic acid/BSA mole ratio. Also plotted is the ^{19}F-NMR difluoromethylene ($1/T_2$) of 2.0 mM methoxyflurane (▲) in BSA solution (1.5 mg/ml), plotted as a function of the oleic acid/BSA mole ratio.

mational changes (*N–F* transition) in BSA structure (15) were previously shown to cause a reduction in isoflurane binding and to abolish saturable binding, as demonstrated by gas chromatographic partition studies (5).

Volatile Anesthetic Binding Constant, K_d, by ^{19}F-NMR T_2. The ^{19}F-NMR T_{2p} for each anesthetic was plotted as a function of buffer anesthetic concentration (Fig. 2). T_{2p} was calculated directly from the data in Fig. 1*A*. The K_d of the oleic acid-displaceable binding was determined from the x intercept for methoxyflurane (K_d = 2.6 ± 0.3 mM), halothane (K_d = 1.3 ± 0.2 mM), and sevoflurane (K_d = 4.5 ± 0.6 mM) (± SD of the linear least-squares fit). The isoflurane data were also plotted (K_d = 1.4 ± 0.2 mM).

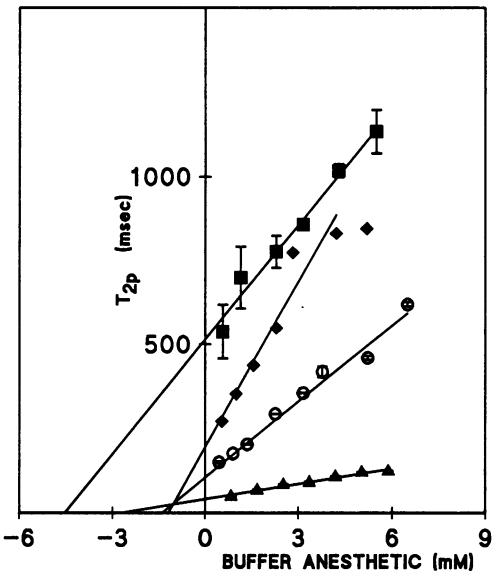

FIG. 2. The ^{19}F-NMR T_{2p} of methoxyflurane (▲), isoflurane (○), halothane (◆), and sevoflurane (■) plotted as a function of anesthetic concentration. The K_d of the oleic acid-displaceable binding is directly evaluated from the x intercept for methoxyflurane (2.6 ± 0.3 mM), isoflurane (1.4 ± 0.2 mM), halothane (1.3 ± 0.2 mM), and sevoflurane (4.5 ± 0.6 mM) (± SD of the linear least-squares fit).

Anesthetic K_i, for Isoflurane Inhibition, by ^{19}F-NMR T_2 Analysis. Methoxyflurane was first tested for its ability to reduce isoflurane binding. With the addition of 2.3 mM methoxyflurane, the trifluoromethyl T_2 of 0.44 mM isoflurane in BSA solution (1.75 mg/ml) was found to double from 102 ± 3 to 192 ± 5 msec. When oleic acid was present (6:1 mole ratio) and 2.3 mM methoxyflurane was added, the T_2 of 0.44 mM isoflurane increased only very slightly from 328 ± 5 to 348 ± 3 msec. Methoxyflurane was therefore predominantly inhibiting oleic acid-displaceable isoflurane binding. T_{2p} was calculated at several isoflurane concentrations in the presence and absence of 2.3 mM methoxyflurane (Fig. 3*A*). The apparent K_d for isoflurane binding to oleic acid-displaceable sites (x intercept) was found to increase. This increase in K_d could result either from an allosteric modulation of isoflurane binding by methoxyflurane or from a direct competitive interaction between methoxyflurane and isoflurane.

To determine if methoxyflurane inhibition of isoflurane binding was of a competitive type, methoxyflurane inhibition of isoflurane binding was characterized at three concentrations of isoflurane. The K_i for isoflurane inhibition was determined by observing the T_2 of isoflurane in the presence of increasing methoxyflurane concentrations. Isoflurane T_{2p} was calculated and plotted for isoflurane concentrations of 0.44, 1.40, and 2.30 mM (Fig. 3*B*), as a function of methoxyflurane concentration. K_i was calculated from the slope according to Eq. 4 (slope = (K_d/K_i) $(T_{2b} + \tau_b)/[R]$). To do this, [R], the oleic acid-displaceable isoflurane binding site concentration, was calculated to be (4.2 ± 0.3 sites) × 26.4 μM BSA = 111 μM. In addition, an isoflurane $(T_{2b} + \tau_b)$ = 8.3 ± 3 msec was calculated from the slope of Fig. 2 (Eq. 3). By using an isoflurane K_d = 1.4 mM (5), the methoxyflurane K_i at each isoflurane concentration was calculated to be 2.3 ± 0.3, 2.5 ± 0.4, and 2.9 ± 0.4 mM. The K_i was expected to be independent of the isoflurane concentration for a competitive interaction. The K_i values were indeed very similar. Further, the methoxyflurane K_i was virtually identical to the methoxyflurane K_d (2.6 ± 0.3 mM) for binding at oleic acid-displaceable sites. This strongly indicated that methoxyflurane was competing with isoflurane for binding at oleic acid-displaceable domains.

The T_2 of isoflurane increased and approached T_{2oleic} in the presence of high methoxyflurane concentrations. In the presence of the highest methoxyflurane concentration (5.8 mM), T_{2oleic} was found to increase from the original value of 328 ± 5 msec to 368 ± 4 msec. This was interpreted as resulting from a reduction in non-oleic-acid-displaceable isoflurane binding. As outlined in *Materials and Methods*, values for T_{2p} were calculated by using T_{2oleic} measured in the presence of the competing anesthetic. For halothane and sevoflurane, virtually no increase in T_{2oleic} was observed when compared to the original 328 ± 5 msec value—namely, 346 ± 4 and 332 ± 5 msec. For these anesthetics, the reduction in isoflurane binding was attributable entirely to oleic acid-displaceable binding.

To confirm that the volatile anesthetics were binding competitively, K_i values were measured for halothane and sevoflurane inhibition of isoflurane binding (Fig. 4) and compared with the directly measured K_d values (Fig. 2). The K_i of halothane (1.3 ± 0.3 mM) was lower than that of methoxyflurane and was identical to the directly determined K_d = 1.3 ± 0.2 mM for halothane binding. The K_i of sevoflurane was 5.4 mM ± 0.7 mM, in agreement with a directly determined K_d = 4.5 ± 0.6 mM. Finally, *n*-octanol, because of its structural resemblance to the hydrocarbon backbone of oleic acid, was tested for its ability to displace isoflurane binding (Fig. 4). A measured K_i of 91 ± 6 μM was

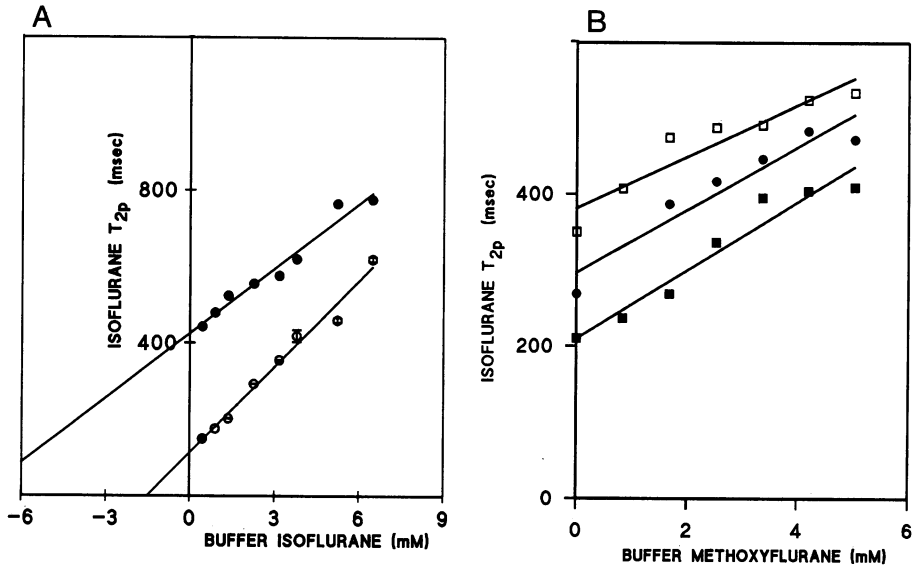

FIG. 3. (*A*) The ^{19}F-NMR isoflurane T_{2p} in the presence (●) or absence (○) of 2.3 mM buffer methoxyflurane. Notice the apparent shift in K_d in the presence of methoxyflurane. (*B*) The ^{19}F-NMR isoflurane T_{2p} at an isoflurane concentration of 0.44 mM (■), 1.4 mM (●), and 2.3 mM (□) in fatty acid-free BSA solution (1.75 mg/ml), plotted as a function of buffer methoxyflurane concentration (n = 1). A methoxyflurane K_i for isoflurane inhibition is directly calculated from each slope (2.3 ± 0.3 mM, 2.5 ± 0.4 mM, and 2.9 ± 0.4 mM) (± SD of the linear least-squares fit).

compared with the literature K_d value for octanol–BSA binding, K_d = 70 μM (16).

DISCUSSION

The fatty acid-binding domains are probable sites of volatile anesthetic interaction with albumin. When oleic acid was added to the BSA solution at a 6:1 mole ratio, the binding of each volatile anesthetic was reduced to a residual, nonsaturable binding component. The identical concentration dependence of oleic acid inhibition of anesthetic binding indicates that each anesthetic binds to BSA in a very similar way. There are at least six predominant long-chain fatty binding domains on albumin (17). The fatty acid-binding sites are nonequivalent and known to allosterically interact with each other (17). It is unclear how many of the fatty acid binding sites are involved in anesthetic binding.

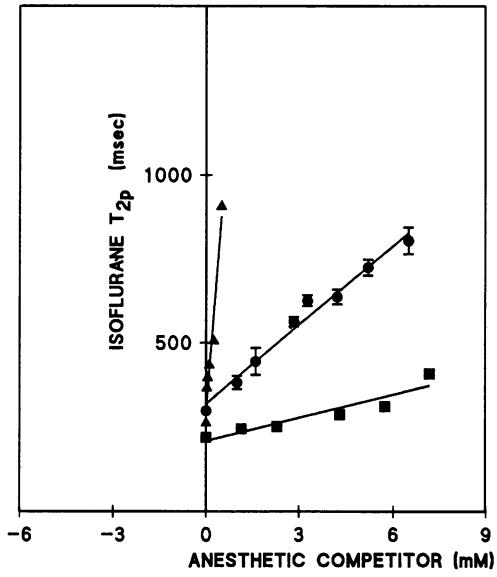

FIG. 4. The ^{19}F-NMR isoflurane T_{2p} at an isoflurane concentration of 1.3 mM in BSA solution (1.75 mg/ml), plotted as a function of anesthetic competitor concentration in buffer—namely, halothane (●), sevoflurane (■), and 1-octanol (▲). The anesthetic K_i for isoflurane inhibition is directly calculated from the slope for halothane (K_i = 1.3 ± 0.2 mM), sevoflurane (K_i = 5.4 ± 0.5 mM), and 1-octanol (K_i = 91 ± 6 μM) (± SD of the linear least-squares fit).

The binding sites for isoflurane (K_d = 1.4 mM, B_{max} = 4.2 sites) (5) are also probably nonequivalent and also may interact allosterically with each other. Isoflurane binding was displaced by other volatile anesthetics to a level similar to that seen with oleic acid. This indicated a significant overlap of anesthetic binding sites. In fact, each of the fluorinated anesthetics reduced isoflurane binding, with a K_i for isoflurane inhibition that was virtually identical to that anesthetic's K_d of binding to oleic acid-displaceable sites. The direct correlation of oleic acid-displaceable anesthetic binding with isoflurane inhibition is strongly suggestive of a direct competition.

Differences in the *in vivo* potency of various volatile anesthetics have generally been attributed to differences in membrane–gas partitioning behavior (18). The current data indicates that different volatile anesthetics can bind at similar site(s) on a protein with different affinities. This suggests that differences in *in vivo* potency may be attributable in part to differences in binding constants and not strictly to lipid partitioning. The volatile anesthetic binding constants for albumin are 3–10 times the concentrations required to produce anesthesia in animals, while the binding constant for octanol is similar to the concentration required for anesthesia. Consequently, the correlation between albumin binding and EC_{50} for anesthesia is not strong. This is not remarkable because albumin is not a target protein responsible for producing anesthesia.

Volatile anesthetics may bind to binding sites for other hydrophobic ligands at concentrations that more closely approximate the levels needed to produce anesthesia. For example the enzyme firefly luciferase is inhibited (presumably by competition for the luciferin binding site) by volatile anesthetics at the same concentrations required to produce anesthesia (1). The binding of volatile anesthetics to fatty acid sites on albumin may also yield some clues as to the actual molecular target sites of volatile anesthetics. For example, the interaction of volatile anesthetics with fatty acid-binding sites on other proteins may be relevant to anesthetic action,

since fatty acids (and arachidonate metabolites) have been shown to directly regulate certain neuronal and smooth muscle ion channel function (10, 19–20).

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