NMR Study of Volatile Anesthetic Binding to Nicotinic Acetylcholine Receptors

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ABSTRACT New lines of evidence suggest that volatile anesthetics interact specifically with proteins. Direct binding analysis, however, has been largely limited to soluble proteins. In this study, specific interaction was investigated between isoflurane, a clinically important volatile anesthetic, and membrane-bound nicotinic acetylcholine receptors (nAChRs) from *Torpedo electroplax*, using ^{19}F nuclear magnetic resonance spectroscopy and gas chromatography. The receptors were reconstituted into 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid vesicles. After correcting for nonspecific partitioning into the lipid, the equilibrium dissociation constant, K_d, of isoflurane binding to nAChR at 15°C was found to be 0.36 ± 0.03 mM. This value is within the clinically relevant concentration range of the agent. Based on the receptor concentrations in the vesicle suspension assayed by the bicinchoninic acid method and the fraction of bound isoflurane, X_b, determined by gas chromatography, an estimate of an average of 9–10 specifically bound isoflurane molecules can be made for each receptor, or two for each subunit. Upon binding, the transverse relaxation time constant (T_2) of ^{19}F resonance of isoflurane is decreased by nearly three orders of magnitude, indicating a dramatic reduction in the mobility of specifically bound isoflurane. Kinetic analysis reveals that the off rate of binding, k_{-1}, is $1.7 \times 10^4\ s^{-1}$. The on rate, k_{+1}, can thus be calculated to be $\sim 4.8 \times 10^7\ M^{-1}\ s^{-1}$, suggesting a nearly diffusion-limited association. This is in contrast to anesthetic binding to a soluble protein, bovine serum albumin (BSA), where k_{+1} and k_{-1} are at least an order of magnitude slower. It is concluded that the presence of lipids may be critical for the correct evaluation of binding kinetics between volatile anesthetics and neuronal receptors.

INTRODUCTION

There is a growing consensus that general anesthetics interact with neuronal proteins (Eckenhoff and Johansson, 1997; Franks and Lieb, 1994). Electrophysiological studies have indicated that a superfamily of neurotransmitter-gated ion channels, including the nicotinic acetylcholine receptors (nAChRs), γ-aminobutyric $acid_A$ ($GABA_A$) receptors, glycine receptors, and 5-hydroxytryptamine ($5\text{-}HT_3$) receptors, is particularly sensitive to general anesthetics (Franks and Lieb, 1996). Site-directed mutagenesis has implicated the existence of an inhibitory site within the aqueous pore of nAChR (Forman et al., 1995) and a potentiating site at the extracellular interfacial regions of transmembrane domains 2 and 3 (MII-MIII) on the glycine and $GABA_A$ receptors (Mihic et al., 1997). Although these studies clearly indicate the involvement of these critical residues in anesthetic action, it remains unknown whether direct anesthetic binding to the receptor is involved in the anesthetic action.

Indeed, experimental results on binding between volatile anesthetics and membrane proteins are scarce. Photoaffinity labeling of [^{14}C]halothane, a clinically important inhalational anesthetic, to native *Torpedo* membranes and isolated nAChR (Eckenhoff, 1996b) suggests that halothane binding to nAChR is saturable and that the conformational changes associated with receptor function and desensitization do not alter the binding domain for halothane. Moreover, while [^{14}C]halothane incorporation demonstrates little subunit selectivity, the labeling pattern within α-subunits (presumably also within other subunits) suggests that most binding occurs at the four putative transmembrane segments, MI–MIV. Whether halothane penetrates between transmembrane sequences to produce significant labeling of the putative pore-lining MII segments remains unclear. The kinetics of binding of volatile anesthetics to transmembrane proteins are largely unknown.

Using ^{19}F nuclear magnetic resonance (NMR) spectroscopy and gas chromatography (GC), we analyzed the binding kinetics of isoflurane, currently the most important clinical anesthetic, to nACh receptors that were purified and reconstituted in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) membranes. Based on a two-site exchange model between nonspecific and specific binding, chemical shift and transverse relaxation time (T_2) measurements were used to determine the equilibrium dissociation constant, K_d, and the dissociation rate constant, k_{-1}, of isoflurane binding to nAChR.

MATERIALS AND METHODS

Previously documented procedures (Ellena et al., 1983) for reconstituting nAChR into DOPC were adapted as follows. Briefly, the electric organ of *Torpedo nobiliana* (Biofish Associates, Georgetown, MA) was freshly dissected and processed (Chak and Karlin, 1992) to obtain nAChR-rich membranes, which were frozen at −70°C until use. For purification, nAChR-rich membranes from 1 kg of electric organ were thawed and

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suspended in 600 ml buffer A (pH 7.4), containing 100 mM NaCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 mM EDTA, and 0.02% sodium azide. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The nAChR-rich membrane suspension was stirred for 2 h in the presence of 1% (w/v) cholate to solubilize the receptors. The mixture was centrifuged at 256,000 $\times$ *g* for 30 min, and the supernatant layer was collected. The solubilized receptors were divided into two equal portions, and each was gently stirred for 2 h with 50 ml AffiGel-10 (Bio-Rad Laboratories, Hercules, CA) that was freshly derivatized with bromoacetylcholine bromide (Research Biochemical International, Natick, MA). The receptor-containing AffiGel-10 was then packed into a 2.5-cm-diameter glass column and washed at a rate of 100 ml/h with 1% cholate in buffer A for 2 h, followed by 0.003% DOPC (Avanti Polar Lipids, Alabaster, AL) and 0.5% cholate in buffer A for an additional period of 2 h. The nAChRs were then eluted with buffer A containing 20 mM carbamylcholine chloride, 0.003% DOPC, and 0.5% cholate. The protein-rich fractions were pooled and dialyzed for 12 h against six changes of 6 liters of buffer A. The membrane suspension was then concentrated to a few milliliters using Centriprep-50 (Amicon Inc., Bevery, MA) and stored at −70°C until use. Immediately before NMR experiments, the reconstituted nAChR-rich membrane suspension was subjected to six cycles of a freeze-thaw process alternating between liquid nitrogen and room temperature to adjust the vesicle size. After the precipitation of large vesicle aggregates, the protein content in the homogeneous vesicle suspension was determined by the bicinchoninic acid method.

All NMR experiments were conducted using a Chemagnetics CMXW-400SLI spectrometer (Fort Collins, CO), operating at 377.168 MHz for ^{19}F resonance. Each NMR sample consisted of a freshly thawed 750-μl DOPC vesicle suspension in a 5-mm-diameter high-precision NMR tube (Wilmad Glass Co., Buena, NJ) with a total volume of 2500 μl (i.e., a vapor space of 1750 μl). NMR samples were prepared in pairs of the same DOPC concentrations and vesicle sizes, but with and without nAChR. The temperature was maintained at 15°C so that the receptor samples could be stable throughout the experiments. Isoflurane was added to the NMR samples with a microsyringe (Hamilton) in steps ranging from 0.03 to 0.2 μl. The equilibrium isoflurane concentrations in the membrane suspension were determined experimentally by NMR, with reference to one of two external standards containing 0.50 mM and 2.19 mM trifluroacetic acid (TFA) in 5- and 10-mm NMR tubes, respectively. The 10-mm standard was used coaxially with the 5-mm sample tube during concentration calibration. The external TFA resonance also served as a frequency reference for chemical shift measurements.

If δ and δ_f are the ^{19}F resonance frequencies of isoflurane in DOPC vesicle suspension with and without nAChR, respectively, and δ_b is the limiting resonance frequency for a hypothetical situation in which all isoflurane molecules were bound to nAChR, then, for a rapid exchange model between free and bound isoflurane, the mole fraction of isoflurane bound to nAChR, X_b, is given by (Xu et al., 1996)

$$X_b = \frac{\delta - \delta_f}{\delta_b - \delta_f} \tag{1}$$

From the definition of K_d, it can be shown that

$$[A]_0 = \frac{[R]_0}{X_b} - K_d = \frac{(\delta_b - \delta_f)[R]_0}{\delta - \delta_f} - K_d \tag{2}$$

or

$$\frac{1}{\delta - \delta_f} = \frac{1}{(\delta_b - \delta_f)[R]_0}([A]_0 + K_d) \tag{3}$$

where $[A]_0$ and $[R]_0$ are the total anesthetic and receptor concentrations, respectively. Thus, in a two-site free exchange model, expressing $1/(\delta - \delta_f)$ as a function of the total isoflurane concentration will yield a straight line, with an x-intercept of $-K_d$. It should be noted that this chemical shift method for the determination of K_d does not require prior knowledge of X_b.

The binding K_d can also be determined by transverse relaxation time (T_2) measurements based on the rapid exchange model given by the following equation (Dubois and Evers, 1992; Xu et al., 1996):

$$T_{2p} = \left(\frac{1}{T_2} - \frac{1}{T_{2f}}\right)^{-1} = \frac{T_{2b} + \tau_b}{[R]_0}([A]_0 + K_d) \tag{4}$$

where $[A]_0$ is the total isoflurane concentration at which T_2 is measured, and K_d is given by the negative intercept on the *x* axis. A modified Carr-Purcell-Meiboom-Gill (CPMG) spin-echo method (Meiboom and Gill, 1958) was used, in which T_2 was measured by incrementing the number of 180° pulses in a pulse train and acquiring a series of free induction decays at the end of each pulse train. After Fourier transformation, the spectral intensities (measured as area integration under the peaks) were fitted to an exponential decay function with respect to the time after the initial 90° pulse. Parallel T_2 measurements were made in paired samples of the same DOPC concentrations and vesicle size, but with or without nAChR. The samples without the receptors were used to determine T_{2f}, the T_2 in the absence of receptor binding.

To determine the binding kinetics, T_2 was also measured as a function of spin-echo time, τ_{cp}. The modified CPMG method overcame the hardware limitation at very short τ_{cp} values, so that measurements can be made at 180°-180° interpulse delays as short as 10 μs.

It has been shown (Allerhand and Gutowsky, 1965; Dubois and Evers, 1992; Xu et al., 1996) that in the presence of receptor binding, T_2 is given by

$$\frac{1}{T_2} = \frac{1 - X_b}{T_{2f}} + \frac{X_b}{T_{2b} + \tau_b} + \tau_b X_b (1 - X_b)(\delta\omega)^2 f(\tau_{cp}) \tag{5}$$

where T_{2b} is the T_2 of isoflurane in the receptor-bound state, $\delta\omega = 2\pi(\delta_b - \delta_f)$, τ_b is the lifetime of isoflurane in the bound state, and $f(\tau_{cp})$ is a hyperbolic tangent:

$$f(\tau_{cp}) = 1 - \frac{2\tau_b}{\tau_{cp}} \tanh \frac{\tau_{cp}}{2\tau_b} \tag{6}$$

We measured X_b by using GC (Xu et al., 1996), and determined $\delta\omega$ from chemical shift measurements (Eq. 1). Nonlinear regression of measured $1/T_2$ as a function of $1/\tau_{cp}$ (Eqs. 5 and 6) will yield the best estimates of T_{2b} and τ_b. The off-rate constant of binding, k_{-1}, equals $1/\tau_b$ by definition, and the on-rate constant, k_{+1}, can be calculated by k_{-1}/K_d.

All fitting parameters from linear and nonlinear regression will be reported as "best estimate $\pm$ standard error of estimate" (Glantz, 1992).

RESULTS

Three separately prepared batches of reconstituted nAChR-rich membranes were used for repeated measurements. The receptor concentrations, as determined by the bicinchoninic acid method for the homogenous nAChR-rich vesicle suspensions, were 5.8, 10.7, and 14.0 μM, respectively. Receptors reconstituted as described above have been shown previously to retain all of their functional properties (Ellena et al., 1983; Chak and Karlin, 1992), including specific activity for toxin binding and ion flux properties. Figure 1 plots the reciprocal of the frequency change for the trifluoromethyl (-CF_3) and difluoromethyl (-CHF_2) resonance as a function of isoflurane concentration. Notice the difference

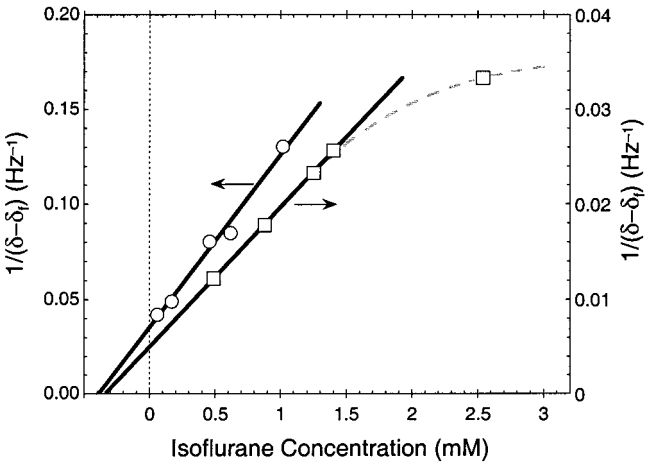

FIGURE 1 Determination of the dissociation constant, K_d, by measurements of NMR resonant frequencies as a function of isoflurane concentration. Data at concentrations lower than 1.5 mM are fitted by linear least-squares regression based on a two-site exchange model (Eq. 3). Extrapolation of the linear fit to the x axis yields $-K_d$. ○, -CF_3 resonance, the left y axis; □, -CHF_2 resonance, the right y axis.

in the scales of the two y-axes, reflecting a larger frequency change due to the higher sensitivity of the -CHF_2 resonance to isoflurane concentration. The solid lines are linear least-squares fits to the data at isoflurane concentrations lower than 1.5 mM. Linear extrapolation to the x axis (Eq. 3) yields a K_d of 0.38 ± 0.06 mM (mean ± SE) from the -CF_3 resonance and 0.34 ± 0.01 mM from the -CHF_2 resonance. The average of the two gives a K_d of 0.36 ± 0.03 mM (mean ± SD). At higher isoflurane concentrations, the data deviated from the linear relationship predicted by Eq. 3, indicating a certain degree of saturation at the receptor binding sites. At the nearly saturating concentration of 2.5 mM, the -CHF_2 resonance of isoflurane shifted by 30 and 65 Hz in samples with 5.8 and 14 μM nAChR, respectively. For the latter, the X_b was determined by six independent GC measurements to be 0.05 ± 0.03 (mean ± SEM, $n = 6$). Using Eq. 1 and $\delta - \delta_f = 65$ Hz, it can be estimated that $\delta_b - \delta_f \approx 1300$ Hz. Because of strong partitioning of isoflurane in lipids, which are used as the control, X_b measurements by GC in samples with lower nAChR concentrations were difficult. However, if one assumes that $\delta_b - \delta_f$ for the same resonance does not vary with receptor concentrations, then X_b for the sample with 5.8 μM nAChR can be estimated to be 0.023 by using Eq. 1. The results are summarized in Table 1.

Fig. 2 shows stack plots of representative ^{19}F spectra, acquired with the modified CPMG pulse sequence from samples without (*A*) and with 5.8 μM nAChR (*B*). The τ_{cp} for Fig. 2, *A* and *B* was 2 ms and 0.01 ms, respectively, as indicated by the labels next to the first (*bottom*) spectrum in each stack. The subsequent spectra in the stack plots are acquired at the multiples of τ_{cp}. Thus, spectra in Fig. 2 *A* were acquired after 1, 2, 3, 4, 5, 6, and 7 refocus pulses in 2-ms intervals, and those in Fig. 2 *B* were acquired after 1, 20, 30, 40, 50, 60, and 70 refocus pulses in 0.01-ms intervals. Spectral line broadening due to the presence of receptors is apparent by comparing spectra in Figs. 2, *A* and *B*. The T_2 at each τ_{cp} value was determined by fitting the spectral intensities, such as those in Fig. 2, to an exponential decay function with respect to the spin-echo time. In the receptor-free lipid vesicle suspensions, the T_2 values of the -CF_3 and -CHF_2 resonance were found to be ~330 ms and ~10 ms, respectively, and were independent of τ_{cp} and isoflurane concentration. Figure 3 shows the dependence of -CF_3 T_2 on isoflurane concentration in the presence of 10.7-μM nAChR. At low isoflurane concentrations, the T_2 dependence fulfills the linear relationship predicted by Eq. 4. This relationship, however, is violated at higher isoflurane concentrations. The solid line at concentration greater than 0 mM shows the best fit to the data using the saturation equation $[a + bx/(c + x)]$, yielding $a = 1.81 \pm 0.13$ ms, $b = 1.15 \pm 0.18$ ms, and $c = 0.22 \pm 0.13$ mM. The first derivative of the fitting curve with respect to concentration at 0 mM defines the slope for the linear extrapolation, resulting in a K_d of 0.35 ± 0.28 mM from the negative intercept on the x axis.

Figure 4 depicts the $1/T_2 \sim 1/\tau_{cp}$ dependence of the -CHF_2 resonance for 2.5 mM isoflurane in the presence of 5.8 and 14.0 μM nAChR. The solid lines show the three-parameter (i.e., τ_b, T_{2b}, and $\delta_b - \delta_f$) nonlinear regression using Eq. 5. The X_b and $\delta_b - \delta_f$ values listed in Table 1 were used as the initial estimates. The regression was rapidly converged to give a $\delta_b - \delta_f$ value of 1329 ± 183 Hz and 1368 ± 238 Hz for the samples with 5.8 μM and 14.0 μM nAChR, respectively. Both values are in excellent agreement with the result of ~1300 Hz obtained from the independent GC and chemical shift measurements (Table 1). The best estimates for τ_b and T_{2b} are, respectively, 54.6 ± 5.6 (mean ± SE) and 29.3 ± 5.1 μs in the presence of 5.8 μM nAChR, and 62.0 ± 7.7 and 32.1 ± 6.7 μs in the presence of 14.0 μM nAChR. Note that although T_2 values for the two samples differ greatly because of the difference in receptor concentration, the τ_b and T_{2b} values are essentially the same within the experimental error, indicating the robustness of the approach. Table 2 compares the averaged kinetic and binding constants of isoflurane binding to nAChR and to a soluble protein, bovine serum albumin (BSA) (Xu et al., 1996).

TABLE 1 Measured and calculated X_b and $\delta_b - \delta_f$ (−CHF_2) for 2.5 mM isoflurane

nAChR (μM)	DOPC (mM)	$\delta - \delta_f$ (Hz) (NMR)	X_b (GC)	$\delta_b - \delta_f$ (Hz) (Eq. 1)	X_b (Eq. 1)
5.8	1.5	30	—	—	0.023
14	1.7	65	0.050	1300	—

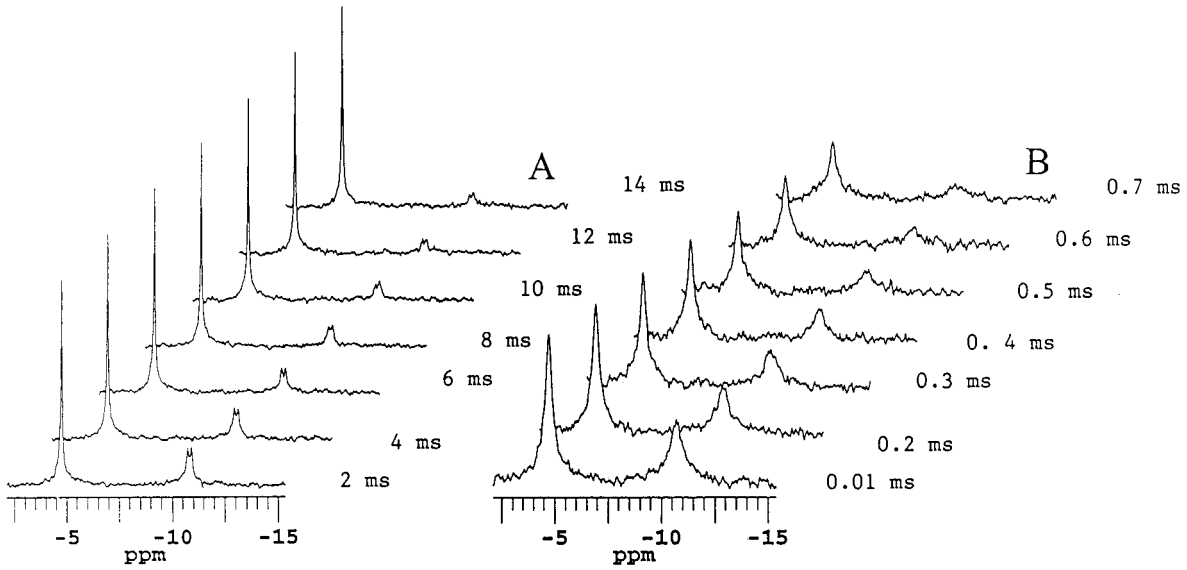

FIGURE 2 Representative stack plots of ^{19}F-NMR spectra of isoflurane acquired in the absence (*A*) and presence (*B*) of nAChR in DOPC vesicles. The first (*lowest*) spectrum in each stack was acquired after a single refocusing pulse at the spin-echo time of τ_{cp} as labeled. The subsequent spectra in the stack were acquired after multiple refocusing pulses in equal intervals of τ_{cp}. Notice the significant line broadening due to the presence of nAChR.

DISCUSSION

A practical challenge faced in studies of volatile anesthetic interaction with membrane proteins is to distinguish specific interaction with the protein from nonspecific interaction with the surrounding lipids. The high sensitivity to nAChR exhibited by both the resonant frequencies and T_2 values of the isoflurane ^{19}F-NMR allows for quantitative analysis of binding kinetics that can be directly attributed to the presence of the receptors in the lipids. The observed association and dissociation constants, therefore, provide direct measures of specific binding that occurs either directly on the receptors themselves, or at particular domains created at the interface between the receptor and the lipids.

The K_d of specific binding determined in Fig. 1 is within the clinical concentration range for isoflurane and comparable to the value of 0.18 ± 0.04 mM for halothane binding

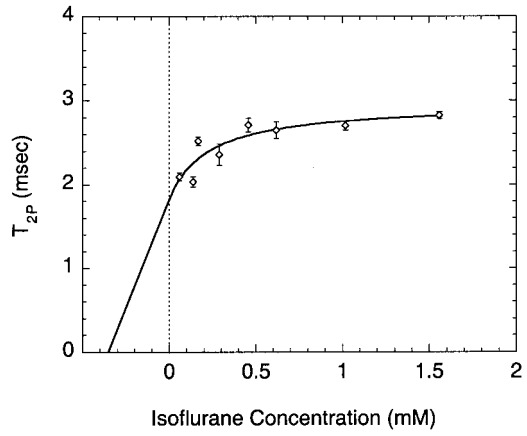

FIGURE 3 Determination of the dissociation constant, K_d, by ^{19}F T_2 measurements of the -CF_3 resonance as a function of isoflurane concentration. The τ_{cp} was set at 25 μs. The error bars show the standard error associated with the exponential fitting of individual T_2 curves. The solid line at a concentration greater than 0 mM shows the nonlinear regression, using a saturation function, $[a + bx/(c + x)]$. The value of the first derivative of the regression curve at 0 mM was used as the slope for linear extrapolation in K_d determination, according to Eq. 4.

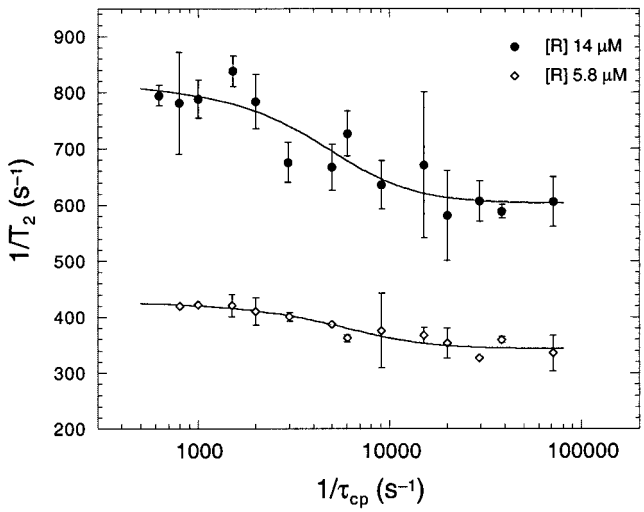

FIGURE 4 Dependence of $1/T_2$ on $1/\tau_{cp}$ is used to determine τ_b and T_{2b}. The error bars show the standard errors of mean of multiple measurements (n = 2–5) at each given τ_{cp}. Where there are no error bars, the experiments were performed only once at those points. The solid lines are best fit to the data, using Eq. 5. The rate constants from the fitting are summarized in Table 2.

TABLE 2 Comparison of isoflurane binding constants to nAChR and BSA

	K_d (mM)	τ_b (μs)	T_{2b} (μs)	k_{-1} (s^{-1})	K_{+1} (M^{-1} s^{-1})
nAChR	0.36	58.2	30.8	1.7×10^4	4.8×10^7
BSA*	1.3	281	4400	3.6×10^3	2.7×10^6

*Taken from Xu et al. (1996).

to nAChR obtained by photoaffinity labeling (Eckenhoff, 1996b). Although the T_2 method for K_d determination (Eq. 4) yielded a similar K_d value by linear extrapolation at very low isoflurane concentrations (Fig. 3), the method showed a lower apparent saturation concentration than the chemical shift method. At first glance, this is rather unexpected because the T_2 method is supposed to be more sensitive to isoflurane concentration, given the significant difference between T_{2b} and T_{2f}. The linear relationships predicted by both the chemical shift (Eq. 3) and T_2 (Eq. 4) methods are derived from a two-site rapid exchange model. Whether a given exchange rate can be considered to be rapid depends on the method of measurements. For isoflurane binding to nAChR, we estimated that the exchange rate is on the order of 10^4 to 10^5 s^{-1} based on the τ_b value. This rate is much greater than the limiting chemical shift difference ($\sim 10^3$ s^{-1}, see Table 1) between the bound and free states, but comparable to the difference between $1/T_{2b}$ and $1/T_{2f}$ ($\sim 10^4$ s^{-1}, see Table 2). Therefore, the rapid exchange condition is fulfilled for the chemical shift method but only marginally for the T_2 method. It should be noted that in the extreme of rapid exchange, the T_2 relaxation process is governed by the fast-relaxation component because the exchange allows all spins to experience the environment causing the fast relaxation. The measured T_2 is then an average of two T_2 components weighted by X_b. At the other extreme when the exchange is slow, one would observe two distinct relaxation components, or biexponential decay. In such a case, T_2 measurement would be dominated by the slow-relaxation (i.e., sharp) component. For isoflurane binding to nAChR measured in this study, we have an intermediate situation in which exchange is sufficient to average two relaxation components into a single exponential decay, but insufficient to completely remove the dominance of the sharp spectral component in the T_2 measurements. Thus, unless the isoflurane concentration is significantly smaller than K_d, the measured T_2 is biased by the narrower spectral component in the intermediate exchange regime. The apparent early saturation shown in Fig. 3 as compared to Fig. 1 reflects the deviation from the rapid exchange model, in addition to the possible saturation at a receptor site. In contrast, because the exchange rate is much greater than the limiting frequency difference, the deviation from the linear relationship in Fig. 1 reflects the true saturation at the receptor binding sites (i.e., X_b becomes invariant at high isoflurane concentrations).

There are other practical concerns that favor the chemical shift method over the T_2 method for K_d determination of anesthetic binding to membrane-associated proteins. For soluble proteins in large quantities, the T_2 method is reliable (Dubois and Evers, 1992; Xu et al., 1996) as long as the difference between $1/T_{2b}$ and $1/T_{2f}$ is much smaller than the exchange rate (i.e., in the rapid exchange regime). However, with membrane-associated proteins where high protein concentration is difficult to attain, the T_2 method is critically dependent on the T_2 values at low isoflurane concentrations, at which the measurements are less accurate and are time consuming. Sample stability is also of some concern when T_2 data acquisition becomes too long. The apparent early saturation (Fig. 4) due to a biased weighting of the narrower spectral component ($1/T_{2f}$) limits the use of T_2 values at higher isoflurane concentrations for K_d determination. In contrast, even at very low anesthetic concentrations, chemical shift can be measured rapidly and accurately. Another advantage of using the method of Eq. 3 is that K_d can be determined solely by chemical shifts, independent of X_b. Thus, it is preferable to use the chemical shift method of Eq. 3, rather than the T_2 method of Eq. 4, for K_d determination of anesthetic binding to membrane proteins.

From X_b and initial receptor concentrations (Table 1), it can be estimated that there are on average 9–10 specifically bound isoflurane molecules per nACh receptor. Assuming that the binding of isoflurane to nAChR resembles that of halothane in that subunit selectivity is asbent (Eckenhoff, 1996b), then, for a pentameric channel structure of nAChR, each subunit on average has at most one specific binding site for two isoflurane molecules or two specific binding sites for one isoflurane molecule each. It is interesting to note that mutagenesis studies (Mihic et al., 1997) have identified as few as two amino acid residues in glycine or the $GABA_A$ receptor subunit that are essential for the receptor's sensitivity to general anesthetics. Given the photolabeling finding (Eckenhoff, 1996b) that most halothane binding to nAChR is in transmembrane domains, it is conceivable that the saturable isoflurane binding identified in this study also occurs within transmembrane domains.

Although at any given time the majority of isoflurane molecules are unbound, the presence of nAChR in the membrane greatly broadens the line width of the isoflurane resonance (compare stack plots in Fig. 2, *A* and *B*). This indicates that significant immobilization occurs to the bound isoflurane molecules. The exchange between the bound and free states is fast enough to result in a partial averaging effect. The T_2 values observed depend, again, on how rapidly T_2 is measured relative to the characteristic time of the exchange process. As shown in Fig. 4, the dependence of $1/T_2$ on $1/\tau_{cp}$ can be well characterized by the exchange model of Eqs. 5 and 6. The nonlinear regression in Fig. 3 shows that T_{2b}, the T_2 of -CHF_2 resonance of bound isoflurane, is $\sim$30 μs, compared to 10,000 μs for the T_2 of the same resonance in receptor-free lipid vesicle suspensions. The reduction in T_2 by nearly three orders of magnitude suggests that the sites for binding must be rather

structurally suited (or structurally specific) for isoflurane, because isoflurane molecules are significantly immobilized once they are bound. It should be noted that high specificity is not necessarily equivalent to tight binding. In fact, compared with isoflurane binding to BSA (a globular protein), the binding of isoflurane to the transmembrane nAChR is nearly diffusion limited, as revealed by our analysis of binding kinetics. As shown in Table 2, both the on- and off-rate constants are an order of magnitude faster for isoflurane binding to nAChR than to BSA. One possible interpretation is that the anesthetic binding sites are more easily accessible from the lipid phase to nAChR than from the aqueous phase to BSA. We showed previously that anesthetic binding sites are amphipathic in character (Xu and Tang, 1997; Tang et al., 1997; 1999a,b; Xu et al., 1998). In globular proteins, such amphipathic sites may be special folds (Eckenhoff, 1996a; Johansson et al., 1999; Franks et al., 1998) with hindered access directly from the aqueous phase. In contrast, in transmembrane proteins, the two-dimensional lateral diffusion in the lipid bilayer may provide an effective passage and orienting device for anesthetic binding to receptor sites. Thus, our finding of rapid binding kinetics for isoflurane to nAChR may suggest that the presence of lipids is important for realistically and accurately evaluating the binding kinetics between volatile anesthetics and neuronal receptors.

In summary, isoflurane binding to nAChR is specific and saturable. There are at most two specific binding sites for each subunit in a pentameric receptor, assuming that the binding has no subunit preference. The motion of the bound isoflurane molecules is greatly restricted, as judged by a decrease in T_2 upon binding by nearly three orders of magnitude. The binding to nAChR is nearly diffusion limited, in contrast to previous findings with soluble proteins. Thus, the presence of lipids probably contributes to the rapid kinetics of binding.

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