Isoflurane Increases the Apparent Agonist Affinity of the Nicotinic Acetylcholine Receptor

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Background: Volatile general anesthetics increase agonist-mediated ion flux through the gamma-aminobutyric acid, glycine, and 5-hydroxytryptamine, (5-HT,) receptors. This action reflects anesthetic-induced increase in the apparent agonist affinity of these receptors. In contrast, volatile anesthetics block ion flux through the nicotinic acetylcholine receptor (nAChR). The authors tested the hypothesis that in addition to blocking ion flux through the nAChR, isoflurane also increases the apparent affinity of the nAChR for agonist.

Methods: Nicotinic acetylcholine receptors were obtained from the electroplax organ of Torpedo nobiliana. The apparent agonist affinity of the nAChR was determined using a new stopped-flow fluorescence assay. This assay derives the apparent agonist affinity of the nAChR from the apparent rates with which agonists convert nAChRs from the resting state to the desensitized state.

Results: Isoflurane significantly increased the apparent affinity (decreased the apparent dissociation constant) of acetylcholine for the nAChR at clinically relevant concentrations. The apparent dissociation constant decreased exponentially with the isoflurane concentration from a control value of 44 ± 4 μM to 1.0 ± 0.1 μM in the presence of 1.5 mM isoflurane, the highest concentration studied.

Conclusions: Isoflurane increases the apparent agonist affinity of the nAChR; however, this effect is poorly resolved in ion flux studies because isoflurane also causes channel blockade. The lack of saturation of isoflurane’s effect on the apparent agonist affinity even at relatively high isoflurane concentrations argues against a single site of anesthetic action. However, it is consistent with isoflurane interactions with several receptor sites that exhibit a range of anesthetic affinities, sites within the membrane lipid, or both. (Key words: Activation; anesthetic mechanism; ion channel; torpedo.)

Although the molecular mechanism and site of action responsible for the anesthetic activities of volatile anesthetics have not been defined, it is generally believed that the anesthetic state results from alterations in neuronal synaptic activity. Consequently, much attention has been focused on a superfamily of structurally and functionally related ligand-gated ion channels that play a critical role in synaptic transmission. Members of this anesthetic-sensitive superfamily include the nicotinic acetylcholine, gamma-aminobutyric acid, (GABA), glycine, and 5-hydroxytryptamine, (5-HT,) receptors.

Electrophysiologic studies of GABA, glycine, and 5-HT3 receptors indicate that at clinically relevant concentrations, volatile anesthetics increase ion flux induced by low concentrations of agonist. Within the context of current kinetic models that describe agonist binding and channel gating, this effect reflects an increase in the apparent affinity of receptors for agonist. Conversely, volatile anesthetics decrease agonist-induced ion flux through the nicotinic acetylcholine receptor (nAChR), presumably because they bind within and block the receptor’s ion channel lumen. A few studies have suggested that in addition to decreasing ion flux, volatile anesthetics may also increase the apparent agonist affinity of the nAChR. Dilger et al. reported that in the presence of very low concentrations of agonist, isoflurane increases both the number of single channel bursts and the peak currents observed from nAChRs expressed in BC3H-1 cells. In addition, we have shown that isoflurane increases the apparent rate with which low concentrations of agonist convert Torpedo nobiliana nAChRs to the desensitized conformational state.

In this study, we tested the hypothesis that isoflurane...
increases the apparent agonist affinity of *Torpedo* nAcChoRs. Because even relatively low concentrations of isoflurane significantly block ion flux, it was first necessary to develop an assay to measure the nAcChoR's apparent agonist affinity that does not depend on measurements of flux. The new sequential mixing stopped-flow fluorescence assay presented in this study determines the apparent affinity of acetylcholine for the nAcChoR from the apparent rates with which acetylcholine induces nAcChoR desensitization (inactivation). We used this assay to study the effect of isoflurane on the apparent agonist affinity of the nAcChoR. Finally, we used a second stopped-flow fluorescence assay to directly measure the effect of isoflurane on the agonist dissociation constant ($K_d$) of the resting-state conformation of the nAcChoR.

**Materials**

*Torpedo nobiliana* was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate, acetylcholine, and carbamylcholine were purchased from Sigma Chemical Company (St. Louis, MO). The fluorescent agonist, 1-(5-dimethylaminonaphthalene)-sulfonamido) n-hexanoic acid $\beta$(N-trimethylammonium bromide) ethyl ester (Dns-C$_6$-Cho), was synthesized according to the procedure of Waksman et al. Isoflurane was purchased from Anaquest (Murray Hill, NJ).

**Methods**

Preparation, Characterization, and Anesthetic Exposure of Nicotinic Acetylcholine Receptor Membranes

Receptor membranes were prepared from the electric organs of *Torpedo nobiliana* in a manner approved by the Massachusetts General Hospital Animal Care and Use Committee. Membranes were stored in *Torpedo* physiologic saline (TPS: 250 mM NaCl, 5 mM KCl, 3 mM CaCl$_2$, 2 mM MgCl$_2$, 5 mM NaHPO$_4$, and 0.02% NaN$_3$, pH 7.0) at $-80^\circ$C and thawed on the day they were used. The number of agonist binding sites was determined from Dns-C$_6$-Cho titrations, as previously described. Acetylcholinesterase activity was inhibited with diisopropylfluorophosphate. Solutions containing isoflurane were prepared from dilutions of isoflurane-saturated TPS assuming a saturated solubility of 15 mM at room temperature. Receptor membranes were mixed with TPS containing the appropriate concentration of isoflurane in a gas-tight syringe to achieve the desired receptor and isoflurane concentrations. Membranes were equilibrated with isoflurane at room temperature for several minutes within the gas-tight glass syringe and then loaded directly into the SX.17 sequential mixing stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, UK) through a Teflon stopcock. Previously we determined that evaporative losses of anesthetic during mixing, equilibration, and transfer are negligible when this technique is used. $^{21}$

Sequential Mixing Stopped-flow Fluorescence Determinations of the Apparent Rate of Agonist-induced Desensitization

Agonists may bind to two classes of sites on the nAcChoR. One class of sites is responsible for channel activation and is located on the two $\alpha$-subunits (1 site per $\alpha$-subunit; two sites per receptor) of the nAcChoR. The other class of sites (presumably one site per receptor) inhibits ion flux, perhaps by binding within and occluding the open channel. $^{27}$ Efficacious agonists such as acetylcholine or carbamylcholine have a relatively low affinity for the inhibitory site. Conversely, the poor efficacy of partial agonists may be the result, in part, of a high affinity for the inhibitory site. In a recent study, we observed that the binding of the fluorescent partial agonist Dns-C$_6$-Cho to the nAcChoR inhibitory site was facilitated by the simultaneous addition of channel activating concentrations of agonist. $^{21}$ In that study, the binding of Dns-C$_6$-Cho was detected as an increase in fluorescence intensity on the millisecond time scale. This suggested that Dns-C$_6$-Cho could be a useful probe to detect receptor activation (opening) because the number of activated (non-desensitized) nAcChoRs would be proportional to the amplitude of the fluorescence signal. We reasoned that the rate of agonist-induced desensitization could be determined using a sequential mixing protocol in which nAcChoRs were first preincubated with the agonist for times ranging from 15 ms to several minutes and then mixed with a solution containing both Dns-C$_6$-Cho (10 $\mu$M) and sufficient acetylcholine (1) to activate all remaining resting-state nAcChoRs and (2) to completely inhibit binding to the two activating sites (i.e., 5 mM). Because acetylcholine has a $K_d$ for the inhibitory site of approximately 100 mM (in the absence of a transmembrane potential), it will not significantly compete with Dns-C$_6$-Cho for binding to that site at a concentration of 5 mM. With the sequential mixing protocol, the amplitude of the fluorescence signal observed on mixing with the 10 $\mu$M Dns-C$_6$-Cho/5 mM acetylcho-
line assay solution will be proportional to the number of nAcChoRs that did not become desensitized during the preincubation step. An excitation wavelength of 290 nm was provided by a 150-Watt xenon arc lamp, and the monochromator bandpass was 5 nm. Fluorescence emission greater than 500 nm was measured through a high pass filter. Fluorescence intensity was recorded for 500 ms after the second mixing step (1,000 points). In a typical experiment, three to five individual runs were signal averaged to reduce noise. Data were acquired at 20 ± 0.3°C.

Sequential Mixing Stopped-flow Fluorescence Determinations of the Kinetics of Dns-C₆-Cho Binding to Resting-state Nicotinic Acetylcholine Receptors

Previously we described a technique to measure the affinity of Dns-C₆-Cho to one of the two agonist binding sites responsible for nAcChoR activation. Briefly, membranes were mixed rapidly (1 ms mixing time; 1:1 vol:v ol) with TPS containing Dns-C₆-Cho. After a 50-ms preincubation period to allow Dns-C₆-Cho to bind to the two receptor activating sites, the nAcChoR/Dns-C₆-Cho solution was mixed (1 ms mixing time; 1:1 vol:vol) with 10 μM acetylcholine and Dns-C₆-Cho at half the concentration present in the premix syringe to maintain a constant (total) Dns-C₆-Cho concentration. The second mixing step occurred within the optical cell, where the change in fluorescence intensity was recorded. With this approach, Dns-C₆-Cho that has bound to nAcChoRs during the first (preincubation) mixing step is competitively displaced from these sites by chemical dilution into acetylcholine during the second mixing step. This leads to a decrease in fluorescence emission. Because Dns-C₆-Cho dissociates rapidly from low-affinity resting-state nAcChoRs but slowly from high-affinity desensitized nAcChoRs, two distinct decays are observed. The slow decay reflects Dns-C₆-Cho dissociation from desensitized nAcChoRs, and the fast decay reflects Dns-C₆-Cho dissociation from resting-state nAcChoRs. The amplitudes of the slow and rapid decays are directly proportional to the fraction of nAcChoRs in the desensitized and resting states, respectively, that had bound Dns-C₆-Cho during the 50 ms preincubation period. An excitation wavelength of 290 nm was used and the monochromator bandpass was 5 nm. Fluorescence emission greater than 500 nm was measured through a high pass filter, and fluorescence intensity was recorded for either 10 or 100 s on a logarithmic time base (1,000 points). In a typical experiment, 10 to 15 individual runs were signal averaged to reduce noise. Data were acquired at 10±0.3°C (rather than at 20°C) to better resolve the rapid dissociation of Dns-C₆-Cho from resting-state receptors.

Computer simulations to determine the fraction of nAcChoRs in each conformational state were performed by solving the differential equations that describe the processes of agonist binding and conformational isomerizations in scheme 3 using SCIENTIST for Windows (Micromath, Salt Lake City, UT).

Statistical Analysis

Data points on agonist concentration-apparent desensitization rate curves represent the average of three determinations, and the error bars indicate the standard deviation. The points were fit to a Hill equation in the form

\[ k_{app} = k_{max} \left( \frac{(\text{Agonist})^n}{(\text{Agonist})^n + (K_d^{app})^n} \right) \]

where \( k_{app} \) is the experimentally determined apparent rate of desensitization, \( k_{max} \) is the maximum apparent rate of desensitization induced by high agonist concentrations, \( K_d^{app} \) is the agonist’s apparent \( K_d \) for desensitization, and \( n \) is the Hill coefficient. The reported errors for the fitted parameters are the standard deviations derived from the curve fit.

The dissociation constant of Dns-C₆-Cho to resting-state nAcChoRs was determined from a plot of the amplitude of the fast decay as a function of Dns-C₆-Cho concentration. The data points on this plot are the average of two determinations, and the error bars indicate the range of the data. These data were fit to a Hill equation in the form

\[ \text{Amp} = \text{Amp}_{\text{max}} \left( \frac{(\text{Dns-C}_6-\text{Cho})^n}{(\text{Dns-C}_6-\text{Cho})^n + (K_d)^n} \right) \]

where \( \text{Amp} \) is the amplitude of the fast fluorescence decay, \( \text{Amp}_{\text{max}} \) is the amplitude of the fast fluorescence decay at high Dns-C₆-Cho concentrations, \( K_d \) is the dissociation constant of Dns-C₆-Cho, and \( n \) is the Hill coefficient. The reported errors for the fitted parameters are the standard deviations derived from the curve fit.

Results

Determination of the Apparent Agonist Affinity

Figure 1 shows the fluorescence traces obtained when nAcChoR membranes were preincubated for 5 min with
state nAChRs. Preincubating nAChRs with 3 μM acetylcholine for 5 min induces a conformational isomerization to a desensitized conformational state. Because desensitized receptors do not open when exposed to agonist, the fluorescence enhancement recorded when nAChRs are mixed with the Dns-C6-Cho/acetylcholine solution was reduced substantially. The amplitude of the fluorescence enhancement in each trace was quantitated by fitting it to an exponential equation with a linear component. In nearly all traces, the exponential component accounted for more than 85% of the entire increase in fluorescence observed during the 500-ms signal acquisition time. It was thus determined that desensitizing nAChRs by preincubating them with 3 μM acetylcholine reduced the amplitude of the exponential fluorescence enhancement from 0.429 to 0.149 (arbitrary units).

To determine the time course acetylcholine-induced desensitization, we used our stopped-flow spectrofluorimeter in the sequential mixing configuration. This allowed us to preincubate membranes with 3 μM acetylcholine for periods ranging from just 15 ms to several minutes before mixing with the acetylcholine/Dns-C6-Cho solution. Figure 2A shows the resulting fluorescence traces obtained using a range of preincubation times. The fluorescence amplitude decreased in a preincubation time-dependent manner. Figure 2B shows a plot of the amplitude of the fluorescence enhancement as a function of preincubation time using preincubating acetylcholine concentrations of either 3 μM or 100 μM. A fit of the amplitude data to an exponential equation to obtain the apparent rates of desensitization yielded values of 0.096 ± 0.006 s⁻¹ and 1.5 ± 0.1 s⁻¹ for 3 μM and 100 μM acetylcholine, respectively. For comparison, fig-

![Figure 2A](image1.png)

![Figure 2B](image2.png)

Anesthesiology, V 90, No 1, Jan 1999

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Fig. 1. Fluorescence traces obtained when nicotinic acetylcholine receptor membranes containing 0.4 μM agonist binding sites were preincubated with either Torpedo physiologic saline alone (control) or 3 μM acetylcholine for 5 min and then mixed with 5 mM acetylcholine and 10 μM Dns-C6-Cho (final concentrations). The solid line superimposed over each trace is a fit to an exponential equation with a linear component to obtain the amplitude of the fluorescence enhancement. Either TPS alone (control) or with 3 μM acetylcholine in TPS and then rapidly mixed with 5 mM acetylcholine and 10 μM Dns-C6-Cho (final concentrations). In the control trace, mixing membranes with both 5 mM acetylcholine and 10 μM Dns-C6-Cho produced a large fluorescence signal on the time scale of tens of millisecond. Under our experimental conditions, this signal arises primarily from Dns-C6-Cho that has bound to the inhibitory site on open

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Fig. 2. (A) The fluorescence traces obtained when nicotinic acetylcholine receptor membranes (0.4 μM agonist binding sites) were preincubated with 3 μM acetylcholine for the indicated times and then mixed with a solution containing 5 mM acetylcholine and 10 μM Dns-C6-Cho (final concentrations). The solid line superimposed over each trace is a fit to an exponential equation with a linear component to obtain the amplitude of the fluorescence enhancement. (B) The amplitude of the fluorescence enhancement as a function of acetylcholine-nicotinic acetylcholine receptor preincubation time from a single set of experiments. The curves are fits of the amplitude data to an exponential equation and yield apparent rates of desensitization equal to 0.096 ± 0.006 s⁻¹ and 1.5 ± 0.1 s⁻¹ on preincubation with 3 μM and 100 μM, respectively. For comparison, the change in amplitude of the fluorescence enhancement on preincubation with Torpedo physiologic saline alone (without acetylcholine) is also shown.
**Fig. 3.** The apparent rates of desensitization as a function of either acetylcholine or carbamylcholine concentration. The curves are fits of the data to equation 1, and the results are given in table 1. The membranes contained 0.4 μM in agonist binding sites during the preincubation period. Data points on all figures represent the mean of at least three separate experiments. Error bars on data points indicate the standard deviations.

Figure 2B also shows the amplitude of the fluorescence enhancement as a function of preincubation time when no acetylcholine was present during preincubation (0 μM). In the absence of acetylcholine, little change in the amplitude of the fluorescence enhancement was observed, even with long preincubation times.

Figure 3 plots the apparent rates of desensitization induced by acetylcholine as a function of agonist concentration. We also show the concentration-response curve obtained when the synthetic agonist carbamylcholine was used in the preincubation step. For both agonists, the apparent rate of desensitization increased with agonist concentration before reaching a plateau. For each agonist, this apparent rate data were fit to equation 1. For acetylcholine, the apparent Kₐ for desensitization was 44 ± 4 μM, and that for carbamylcholine was 670 ± 60 μM.

**Fig. 4.** The apparent rates of desensitization as a function of acetylcholine concentration in the presence of 1 mM isoflurane and in the absence of anesthetic (control). The curves are fits of the data to equation 1, and the results are given in table 2. Data points on all figures represent the mean of three separate experiments. Error bars on data points indicate the standard deviations. The inset shows the relative increase in the apparent rates of desensitization induced by 1 mM isoflurane as a function of acetylcholine concentration. The membranes contained 0.4 μM in agonist binding sites during the preincubation period.

Ioflurane 60 μM. The maximal rate of desensitization for both agonists was approximately 2 s⁻¹, and the Hill coefficients were near 1 (table 1). For comparison, table 1 also gives the apparent Kₐ for Torpedo nAcChRo activation by these agonists as measured with a rapid (15 ms) quenched-flow assay.⁴⁹

Figure 4 shows the apparent rates of desensitization induced by a range of acetylcholine concentrations in the presence of 1 mM isoflurane and in the absence of isoflurane (control). Isoflurane produced the greatest relative increase in the apparent rate of desensitization at low acetylcholine concentrations (see the inset of figure 4). At a concentration of 1 mM, isoflurane increased the

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**Table 1. Parameters Derived from Agonist Concentration-apparent Desensitization Curves using Equation 1**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Apparent Kₐ * (μM)</th>
<th>Kₑ (μM)</th>
<th>n</th>
<th>kₘ₅₀ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>49 ± 3</td>
<td>44 ± 4</td>
<td>0.97 ± 0.07</td>
<td>2.00 ± 0.05</td>
</tr>
<tr>
<td>Carbamylcholine</td>
<td>1,000 ± 100</td>
<td>670 ± 60</td>
<td>0.85 ± 0.06</td>
<td>1.96 ± 0.05</td>
</tr>
</tbody>
</table>

* From quenched-flow studies using a 15 ms flux times at 4°C.⁴⁹ Errors are the standard deviations derived from nonlinear least square fitting of data to equation 1.

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Anesthesiology. V 90, No 1, Jan 1999
Table 2. Parameters Derived from Acetylcholine Concentration-apparent Desensitization Curves Using Equation 1 after Equilibration with Isoflurane

<table>
<thead>
<tr>
<th>Isoflurane (mM)</th>
<th>Kd^app (μM)</th>
<th>n</th>
<th>kmax (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44 ± 4</td>
<td>0.97 ± 0.07</td>
<td>2.00 ± 0.05</td>
</tr>
<tr>
<td>0.25</td>
<td>12.5 ± 0.9</td>
<td>1.15 ± 0.08</td>
<td>1.83 ± 0.02</td>
</tr>
<tr>
<td>0.5</td>
<td>7.3 ± 0.5</td>
<td>1.16 ± 0.08</td>
<td>1.82 ± 0.03</td>
</tr>
<tr>
<td>1.0</td>
<td>3.6 ± 0.6</td>
<td>1.2 ± 0.2</td>
<td>1.84 ± 0.06</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>1.97 ± 0.06</td>
</tr>
</tbody>
</table>

Errors are the standard deviations derived from nonlinear least square fitting of the data to equation 1.

The apparent rate of desensitization by 6.2 times in the presence of 1 μM acetylcholine, but it had no detectable effect at high acetylcholine concentrations. This plot also shows that 1 mM isoflurane shifts the acetylcholine concentration-response curve to the left. The apparent Kd of acetylcholine determined using equation 1 was reduced from 44 ± 4 μM to 3.6 ± 0.6 μM. Table 2 shows the effect of a range of isoflurane concentrations on the apparent affinity of the nAChOR for acetylcholine and the kinetics of acetylcholine-induced desensitization. At the lowest concentration of isoflurane studied (0.25 mM), the apparent Kd of acetylcholine for the nAChOR was reduced to 12.5 ± 0.9 μM. By 1.5 mM, the apparent Kd decreased to 1 ± 0.1 μM.

Determination of the Effect of Isoflurane on the Binding of Dns-C6-Cho to the Nicotinic Acetylcholine Receptor

Because our desensitization experiments showed that isoflurane increases the apparent affinity of nAChORs, we tried to determine whether isoflurane increases the microscopic agonist binding affinity (Kd) of resting-state nAChORs; a change in the apparent agonist affinity may reflect alterations in either the receptor's Kd or channel gating kinetics. To characterize agonist binding to the nAChOR resting state, we used a protocol that allows Dns-C6-Cho to bind to the two activating sites of the nAChOR during the preincubation step before being displaced competitively by acetylcholine. We believe that we can characterize Dns-C6-Cho binding to only one of the sites because agonist binding to the other site is thought to occur with such low affinity that Dns-C6-Cho would dissociate from it within the 1-ms mixing time of our spectrophotometer. In these experiments, we used a Dns-C6-Cho-receptor preincubation time of just 50 ms to minimize conversion of receptors from the resting state to the desensitized state during preincubation. A preincubation time of 50 ms is, however, sufficiently long to allow Dns-C6-Cho to equilibrate with sites on resting-state receptors even at Dns-C6-Cho concentrations as low as 0.5 μM. Representative fluorescence traces obtained in the absence of anesthetic and in the presence of 1.5 mM isoflurane are shown in figure 5. Panel A shows the slow time scale over which Dns-C6-Cho dissociates from high-affinity agonist binding sites on desensitized nAChORs when chemically diluted into acetylcholine. Panel B shows the same traces as in panel A, with the first 200 ms expanded to reveal the rapid time scale over which Dns-C6-Cho dissociates from one of the low-affinity binding sites on resting-state nAChORs. A fluorescence enhancement reflecting Dns-C6-Cho binding to the agonist self-inhibitory site is also seen in panel B, because the high, channel-activating acetylcholine concentration used to displace Dns-C6-Cho from affecting Dns-C6-Cho association with the agonist self-inhibition site may be seen on the time scale of ~100 ms. The time scale of Dns-C6-Cho association with the agonist self-inhibition site is slow compared with that seen in figures 1 and 2, because the Dns-C6-Cho concentration is only 2 μM, and the temperature is 10°C rather than 20°C.

Anesthesiology, V 90, No 1, Jan 1999

Fig. 5. The effect of 1.5 mM isoflurane on the fluorescence traces obtained when nicotinic acetylcholine receptor membranes (0.4 μM binding sites) are preincubated with 2 μM Dns-C6-Cho for 50 ms and then mixed with a solution containing 5 mM acetylcholine and 10 μM Dns-C6-Cho (final concentrations). The slow decay reflects Dns-C6-Cho dissociation from desensitized receptors. The same traces are shown in panel B, with the first 200 ms expanded to show the fast fluorescence decay. The fast decay reflects Dns-C6-Cho dissociation from one of the agonist binding sites on the nicotinic acetylcholine receptor resting state. In addition, a fluorescence enhancement reflecting Dns-C6-Cho association with the agonist self-inhibition site may be seen on the time scale of ~100 ms. The time scale of Dns-C6-Cho association with the agonist self-inhibition site is slow compared with that seen in figures 1 and 2, because the Dns-C6-Cho concentration is only 2 μM, and the temperature is 10°C rather than 20°C.
ISOFLURANE POTENTIATES AGONIST ACTIONS ON THE nACCHO

Fig. 6. A plot of the amplitude of the fast fluorescence decay as a function of Dns-C₆-Cho concentration in the presence of 1.5 mM isoflurane. The curve is a fit to equation 2, yielding a dissociation constant (Kₐ) of 1.2 ± 0.2 µM and a Hill coefficient of 1.8 ± 0.4. The Kₐ is not different from that measured in the absence of anesthetic. The nicotinic acetylcholine receptor concentration during the preincubation period was 0.2 µM. Data points on all figures represent the mean of two separate experiments, and the error bars indicate the range of the data.

the agonist activating sites also opens resting-state nACCHO, facilitating Dns-C₆-Cho binding to the agonist self-inhibitory site. A fit of the first 200 ms of each trace in panel B to a double exponential equation revealed that the rate with which Dns-C₆-Cho dissociated from this resting-state site was little affected by isoflurane; Dns-C₆-Cho dissociated from this site at a rate of 208 s⁻¹ in the presence of 1.5 mM isoflurane compared with 225 s⁻¹ in its absence.

Because the amplitude of the fast fluorescence decay reflects the number of resting-state nACChoRs that had bound Dns-C₆-Cho during the 50-ms preincubation period, the affinity of Dns-C₆-Cho to this site may be determined from a plot of the amplitude of this decay as a function of Dns-C₆-Cho concentration. Provided that the concentration of Dns-C₆-Cho is greater than the concentration of agonist binding sites, the Kₐ of Dns-C₆-Cho for this site will be equal to the Dns-C₆-Cho concentration producing a half-maximal fast decay amplitude. Figure 6 shows a plot of the amplitude of the fast decay versus Dns-C₆-Cho concentration. The Kₐ of this site was determined from equation 2 to be 1.2 ± 0.2 µM. This is not different from our previously reported value of 1.1 ± 0.2 µM for the Kₐ of Dns-C₆-Cho in the absence of anesthetic. Finally, because isoflurane did not significantly alter either the dissociation rate constant or Kₐ of Dns-C₆-Cho for this site on resting-state nACChoRs, we can conclude that the association rate constant is also not significantly affected by isoflurane. From the ratio of the dissociation rate constant and the Kₐ, we estimate that the association rate of Dns-C₆-Cho to this resting-state site is approximately 10⁸ M⁻¹s⁻¹.

Discussion

Current Kinetic Models of Nicotinic Acetylcholine Receptor Conformational Transitions

Electrophysiologic, rapid quench-flow, and ligand-binding studies of Torpedo nACChoRs have led to the development of detailed kinetic models such as the one shown in scheme 1 that describe the processes of agonist binding, channel activation, agonist self-inhibition, and desensitization.

Scheme 1

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R <-> AR <-> A<sub>2</sub>R <-> A<sub>2</sub>R<sup>*</sup> <-> A<sub>2</sub>R<sup>*</sup>A
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In the absence of agonist, this scheme considers the nACChoR to exist in a conformational equilibrium between an activatable resting state (R) that has a low affinity for agonist and an inactivatable desensitized state (D) that has a high affinity for agonist. Before agonist-induced conformational transitions, 80% to 90% of all Torpedo nACChoRs are in the resting state, and the remaining 10% to 20% are in the desensitized state. The binding of two agonist molecules (A) to resting-state receptors leads to a conformational transition first to an open channel state (A<sub>2</sub>R<sup>*</sup>) and ultimately to a set of closed, desensitized states. Two agonist molecules may also bind to receptors preexisting in the desensitized state, but this does not lead to channel opening. In addition, one agonist molecule may bind to an agonist self-inhibitory site on the open channel state of the nACChoR (A<sub>2</sub>R<sup>*</sup>A). This site is distinct from the two sites that lead to channel activation and, instead, leads to inhibition of ion flux.

Previous work showed that in the presence of efficacious agonists, nACChoR desensitization proceeds primarily from states A<sub>2</sub>R<sup>*</sup> and A<sub>2</sub>R<sup>*</sup>A. At agonist concen-
trations at which desensitization occurs primarily from the open state and agonist self-inhibition is negligible, scheme 1 may be simplified to

\[ \begin{align*}
A \xrightleftharpoons[r_{d1}]{K_{d1}} R & \quad A \xrightleftharpoons[r_{d2}]{K_{d2}} A_R\quad \phi \quad A_2 R \xrightleftharpoons[r_{des}]{K_{res}} A_2 D
\end{align*} \]

Scheme 2

Agonist binding studies using Torpedo nAChR membranes and analyses of single-channel currents from cloned Torpedo nAChR suggest that $K_{d1}$ and $K_{d2}$, the affinities of the two agonist binding sites on the resting state, differ considerably.\textsuperscript{28,30,35} This difference in affinity largely reflects the difference in the rate with which agonists dissociate from these two sites.\textsuperscript{30} Conversely, their agonist association rates are similar and near the limit of diffusion ($\sim 10^{8}$ $\text{m}^{-1}\text{s}^{-1}$).\textsuperscript{25,28,30,32,35}

Assuming that agonist binding and channel gating kinetics are fast relative to desensitization, scheme 2 predicts that the experimentally determined apparent rate of desensitization will increase with agonist concentration and reach a plateau value equal to the sum of the rate constants for desensitization ($k_{des}$) and resensitization ($k_{res}$).\textsuperscript{34}

Figure 3 clearly shows this behavior. Another critical prediction of scheme 2 is that an agonist's apparent $K_{d}$ for desensitization will be equal to its apparent $K_{d}$ for channel activation. This has been confirmed experimentally for acetylcholine by Forman et al.\textsuperscript{29} and by Forman and Miller\textsuperscript{35} using rapid quenched-flow techniques and is shown in the current study for acetylcholine and carbamylcholine by the near identity of their apparent $K_{d}$ values for ion flux and desensitization (table 1).

The Effect of Isoflurane on Nicotinic Acetylcholine Receptor Conformational Transitions

Figure 7 plots the logarithm of the apparent $K_{d}$ of acetylcholine as a function of isoflurane concentration. A linear fit of these data has a slope of $-1$ mm$^{-1}$, indicating that in the concentration range studied, the apparent $K_{d}$ is reduced by a factor of approximately 10 for each 1 mm isoflurane. We detected no clear evidence that isoflurane's effect on the apparent $K_{d}$ of acetylcholine saturates even at isoflurane concentrations as high as 1.5 mm. This tends to argue against a mechanism in which isoflurane increases the apparent agonist affinity of the nAChR by binding to a single receptor site. However, the data are consistent with isoflurane interactions with several receptor sites exhibiting a range of affinities. Such anesthetic binding sites may be located in cavities between $\alpha$-helical transmembrane domains or at the lipid-protein interface.\textsuperscript{11,36-38} However, we cannot rule out the possibility that the lack of saturation points to a lipid-mediated mechanism. Previous studies using nAChRs reconstituted into defined lipid bilayers have shown that the physicochemical properties of the lipid bilayer do, in fact, modulate channel gating and ion flux.\textsuperscript{39,40} One molecular mechanism proposed by Cantor\textsuperscript{41} suggests that anesthetics may increase $\phi$ in direct proportion to their membrane concentrations by altering the lateral pressure that the lipid bilayer exerts on receptors.

To further examine the effect of a simultaneous isoflurane-induced increase in the apparent affinity of nAChR for acetylcholine and isoflurane-induced channel blockade, scheme 2 was modified to account for the binding of isoflurane to all receptor states\textsuperscript{16,42}.

Scheme 3

![Scheme 3 Diagram](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931264/)
ISOFLURANE POTENTIATES AGONIST ACTIONS ON THE $\text{nAChOR}$

Table 3. Acetylcholine Rate Constants Used for Computer Simulations of Scheme 3

<table>
<thead>
<tr>
<th>Rate Constant</th>
<th>Definition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_1$</td>
<td>Agonist association rate to the higher affinity site on the resting state</td>
<td>$0.6 \times 10^6 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>Agonist dissociation rate from the higher affinity site on the resting state</td>
<td>$250 \text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Agonist association rate to the lower affinity site on the resting state</td>
<td>$1 \times 10^6 \text{M}^{-1}\text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>Agonist dissociation rate from the lower affinity site on the resting state</td>
<td>$40,000 \text{s}^{-1}$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>Channel opening rate constant</td>
<td>$45,000 \text{s}^{-1}$ ($0.0 \text{mm isoflurane}$)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Channel closing rate constant</td>
<td>$2,500 \text{s}^{-1}$ ($0.5 \text{mm isoflurane}$)</td>
</tr>
<tr>
<td>$k_{\text{des}}$</td>
<td>Rate constant for desensitization</td>
<td>$8,000 \text{s}^{-1}$ ($1.0 \text{mm isoflurane}$)</td>
</tr>
<tr>
<td>$k_{\text{res}}$</td>
<td>Rate constant for resensitization</td>
<td>$250 \text{s}^{-1}$ ($1.5 \text{mm isoflurane}$)</td>
</tr>
<tr>
<td>$k_{\text{on}}$</td>
<td>Isoflurane association rate</td>
<td>$2 \text{s}^{-1}$</td>
</tr>
<tr>
<td>$k_{\text{off}}$</td>
<td>Isoflurane dissociation rate</td>
<td>$7.22 \times 10^{-6} \text{s}^{-1}$†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2,000 \text{s}^{-1}$‡</td>
</tr>
</tbody>
</table>

* From single-channel studies using nACChORs from Torpedo expressed in fibroblasts. 
† From Dns-Cp-Cho binding studies using nACChORs from the electric organs of Torpedo. 
‡ From single-channel studies using nACChORs expressed in clonal BC3H-1 cells.

Then we computer-simulated scheme 3 using previously reported values for agonist binding and channel gating kinetics derived from single-channel studies of Torpedo nACChORs (table 3). The rate constant for desensitization used in our simulations was derived from the current study, and the rate constant for resensitization was taken from the study by Raines and Krishnan. Because the binding kinetics of isoflurane to Torpedo nACChORs have not been reported, we used data reported for closely related nACChORs expressed in BC3H-1 cells and assumed that the kinetics of isoflurane binding to its channel site were approximately the same for all states, as suggested by Dilger et al. Our data do not allow us to unequivocally assign the isoflurane-induced increase in the apparent agonist affinity to either a decrease in $K_{\text{on}}$, an increase in $\beta$, a decrease in $\alpha$, or all of these, in scheme 3. However, as a practical matter, we found that our simulations were not very sensitive to any assumptions regarding which of these three parameters was affected by isoflurane. Therefore, for simplicity, we assumed that isoflurane increased acetylcholine's apparent affinity by solely reducing $\alpha$ in the simulations shown in figure 8. Figure 8A shows a simulation of the effects of 1 mM acetylcholine on resting-state nACChORs in the absence of isoflurane. Receptors rapidly bind acetylcholine, and within 300 $\mu$s of agonist exposure, the maximum number of nACChORs are open. Ultimately, these open-state nACChORs isomerize to the desensitized state on the time scale of hundreds of milliseconds. Figures 8B and C show the results of simulations in which nACChORs were preincubated with either 0, 0.5, 1, or 1.5 mM isoflurane and then exposed to constant acetylcholine concentrations of either 1 mM (fig. 8B) or 1 $\mu$m (fig. 8C). For clarity, we have shown only the fraction of nACChORs in the unblocked, open state ($A_{2,R}^*$) in panels B and C. This is the receptor state responsible for ion flux. In the presence of 1 mM acetylcholine, our simulations predict that isoflurane will reduce the maximum number of receptors that reach the unblocked, open state (fig. 8B). With high acetylcholine concentrations, the predominant effect of isoflurane is to block channels rather than to increase the apparent agonist affinity of nACChOR because acetylcholine's channel-activating activity is already near maximal level, even in the absence of isoflurane. This is consistent with the results of many studies showing that isoflurane inhibits ion flux induced by high concentrations of agonist. Conversely, our simulations indicate that isoflurane will increase the maximum number of receptors that reach the unblocked, open state on exposure to 1 $\mu$m acetylcholine (fig. 8C). Under conditions of low agonist occupancy of receptor binding sites, an increase in the apparent affinity will have a significant effect on the maximum number of channels that open. For example, our simulations predict that 0.5 mM isoflurane will increase by 41% the maximum number of channels that reach the open, unblocked state in the presence of 1 $\mu$m acetylcholine. This is reasonably consistent with the results of experiments using nACChORs expressed in BC3H-1 cells that indicate that 1.9% isoflurane increases the current induced by 1 $\mu$m acetylcholine by 25%. In fact, our simulations predict that were it not for channel blockade, 0.5 mM isoflurane would increase the peak current induced by 1 $\mu$m acetylcholine by 320%

Anesthesiology, V 90, No 1, Jan 1999
Fig. 8. Computer simulations of scheme 3 using the parameters in table 3. (A) The relative concentrations of receptors in the unliganded resting (R), singly liganded resting (AR), doubly liganded preopen (A₂R), open (A₂R⁺), and desensitized (A₂D) states after exposure to 1 mM acetylcholine (no isoflurane). (B) The relative concentrations of receptors in the unblocked, open (A₂R⁺) state on exposure to 1 mM acetylcholine after preincubation with the indicated concentrations of isoflurane. This concentration approximates that achieved at the neuromuscular junction at the time of nicotinic acetylcholine receptor activation. (C) The relative concentrations of receptors in the unblocked, open (A₂R⁺) state on exposure to 1 μM acetylcholine after preincubation with the indicated concentrations of isoflurane. The y axis used in C is much smaller than that used in B.

(simulation not shown). For simplicity, we have not modeled the direct desensitizing effects of isoflurane; high concentrations of isoflurane convert resting-state nAChR to the desensitized state even in the absence of agonist. This effect is reflected in figure 5 as an isoflurane-induced increase in the amplitude of the slow decay and a reciprocal decrease in the amplitude of the fast decay. Thus, we expect that our simulations slightly overestimate the peak current. However, this overestimation would be negligible at clinically relevant isoflurane concentrations and less than 20% even at the highest concentration of isoflurane used in this study.

Just as isoflurane potentiates the effects of agonist on the nAChR, other volatile anesthetics have been shown to potentiate the effects of agonist on the other members of the superfamily of ligand-gated ion channels. For example, Jenkins et al. showed that methoxyflurane increases the apparent affinity of 5-HT₁ receptors from neuroblastoma cells in a dose-dependent manner. Electrophysiologic studies by Lin et al. have shown that 1.9 × minimum alveolar concentrations of enflurane and halothane increase the apparent affinity of GABAₐ receptors expressed in oocytes by 1.8 and 1.5 times, respectively. Similarly, Longoni et al. have suggested that the increase in muscimol-induced chloride flux induced by halothane reflects an increase in the agonist's affinity for the GABAₐ receptor. Thus, it seems reasonable to postulate that a similar molecular mechanism underlies the potentiating actions of volatile anesthetics on all members of this superfamily.

The similar effects that volatile anesthetics have on superfamily members may extend beyond their potentiating actions to include their inhibitory actions. Specifically, volatile anesthetics have also been shown to block agonist-induced ion flux through GABAₐ receptors. In contrast to the nAChR, these inhibitory actions are typically evident in the GABAₐ receptor only at high anesthetic concentrations or after exposure to high, desensitizing concentrations of agonist. However, it seems reasonable to consider the possibility that similar to the nAChR, the GABAₐ receptor is simultaneously potentiated and inhibited by volatile general anesthetics, but that the potentiating effect predominates in GABAₐ receptors because (1) its channel-blocking site has a much lower affinity for volatile anesthetics, (2) anesthetic binding to the inhibitory site of the GABAₐ receptor is substantially less effective at blocking flux through the open channel state, or both.

In conclusion, we characterized the effects of isoflurane on the apparent affinity of acetylcholine. Our stud-
isoflurane substantially reduces the apparent $K_v$ of acetylcholine for the nAChR at clinically relevant concentrations. This reduction was anesthetic concentration dependent and failed to show any clear signs of saturating even at isoflurane concentrations as high as 1.5 mA. The similarity in the kinetic mechanism responsible for isoflurane-induced potentiation of nAChRs and other members of the structurally and functionally similar superfamily of ligand-gated ion channels strongly suggests that the underlying molecular mechanism is the same for all members. However, ion channel blockade by isoflurane masks its potentiating effect at the agonist concentrations typically used in electrophysiologic and rapid quench-flow studies. As the only member of the superfamily that can be obtained in quantities sufficient for detailed biochemical and biophysical studies, our studies suggest that the nAChR represents a unique and valuable model for defining the molecular mechanism by which anesthetics increase the apparent agonist affinity of ligand-gated ion channels, even though it may not be a major in vitro target for anesthetic action.

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