Anesthesiology
2000; 92:775–85

Isoflurane Increases the Apparent Agonist Affinity of the Nicotinic Acetylcholine Receptor by Reducing the Microscopic Agonist Dissociation Constant

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Background: **Isoflurane increases the apparent agonist affinity of ligand-gated ion channels. This action reflects a reduction in the receptor's agonist dissociation constant and/or the preopen/open channel state equilibrium. To evaluate the effect of isoflurane on each of these kinetic constants in the nicotinic acetylcholine receptor, the authors analyzed isoflurane's actions on (1) the binding of the fluorescent agonist Dns-C_6-Cho to the nicotinic acetylcholine receptor's agonist self-inhibition site and (2) the desensitization kinetics induced by the binding of the weak partial agonist suberyldicholine.**

Methods: **The dissociation constant for Dns-C_6-Cho binding to the self-inhibitory site was determined using stopped-flow fluorescence spectroscopy. The values of the kinetic constants for agonist binding, channel gating, and desensitization were determined by modeling the suberyldicholine concentration–dependence of the apparent rate of desensitization.**

Results: **Isoflurane did not significantly alter the dissociation constant for Dns-C_6-Cho binding to the self-inhibitory site even at a concentration as high as 1.5 mM, the highest concentration studied. At this concentration, isoflurane substantially reduced the dissociation constant for suberyldicholine binding to its channel opening site by 97% from 17 ± 5 μM to 0.5 ± 0.2 μM, whereas the preopen/open channel state equilibrium was reduced only from 19.1 to 5 ± 1.**

Conclusions: **Isoflurane increases the apparent agonist affinity of the nicotinic acetylcholine receptor primarily by reducing the agonist dissociation constant of the site responsible for channel opening rather than altering channel gating kinetics. (Key words: Activation; anesthetic mechanism; ion channel; partial agonist.)**

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Received from the Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, Massachusetts. Submitted for publication May 19, 1999. Accepted for publication October 7, 1999. Supported in part by a FIRST award from the National Institutes of General Medical Sciences, Bethesda, Maryland (no. GM53481). Presented in part at the annual meeting of the Association of University Anesthesiologists, Pittsburgh, Pennsylvania, May 14, 1999.

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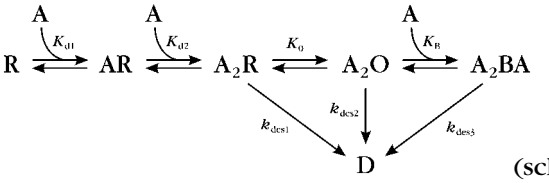
THE nicotinic acetylcholine, γ-aminobutyric $acid_A$ ($GABA_A$), glycine, and 5-$hydroxytryptamine_3$ receptors form a structurally and functionally related superfamily of ligand-gated ion channels.[1–3] Upon agonist binding to a closed resting state, all members of this superfamily undergo a rapid conformational transition to an open channel state(s) that permits ion flux, followed by a slower conformational transition to a closed desensitized state(s).[4–10] The processes of agonist binding and unbinding, channel gating, and desensitization may be most simply represented by the following generic kinetic scheme:

$$\mathrm{R} \underset{K_d}{\overset{\mathrm{A}}{\rightleftharpoons}} \mathrm{AR} \overset{K_o}{\rightleftharpoons} \mathrm{AO} \xrightarrow{k_{des}} \mathrm{AD} \quad \text{(scheme 1)}$$

where A is the agonist, R is the resting state, AR is the preopen state, AO is the open channel state, AD is the desensitized state, K_d is the agonist dissociation constant for the resting state, K_o is the equilibrium constant between the preopen and open channel states ([AR]/[AO]), and k_{des} is the rate constant for desensitization. This simple scheme accounts for the observation that both ion flux and apparent desensitization rates increase before plateauing with agonist concentration and for the existence of partial agonists. Partial agonists are defined as agonists that open few channels even at receptor saturating concentrations ($K_o > 1$), whereas efficacious agonists open most channels at high concentrations ($K_o < 1$).

Volatile general anesthetics increase ion flux and/or apparent desensitization rates induced by low concentrations of agonist, shifting agonist concentration–response curves to the left.[11–16] Within the context of scheme 1, this increase in apparent agonist affinity may reflect a decrease in either K_d or K_o. Unfortunately, direct measurements of K_d and K_o using rapid agonist application techniques are difficult because the rate constants that define these equilibria are typically fast.[7,17,18]

The nicotinic acetylcholine receptor (nAcChoR) from *Torpedo* is the only member of this superfamily that can be obtained in the multimilligram quantities required for detailed biophysical and biochemical analysis. Such studies have led to the development and testing of more precise kinetic schemes that provide a powerful framework for defining the effects of general anesthetics on a ligand-gated ion channel[19]:



(scheme 2)

Scheme 2 for the nAcChoR differs from generic scheme 1 in several important ways. First, two agonist molecules must bind to the nAcChoR to open its channel. Second, an agonist molecule may also bind to the nAcChoR's open channel state at a site that is distinct from those that induce channel opening, leading to inhibition of ion flux (A_2BA). This is referred to as agonist self-inhibition and typically (but not always) occurs at high agonist concentrations.[20] Finally, nAcChoRs may desensitize from all states that have agonist molecules bound to the two sites that cause channel opening (A_2R, A_2O, and A_2BA). Ion flux studies suggest that the rate constants for *Torpedo* nAcChoR desensitization in scheme 2, k_{des1}, k_{des2}, and k_{des3}, are similar and in the range of 2–7 s^{-1}.[21–23] The rates of desensitization from unliganded and singly liganded states and the rates of resensitization are much slower and not considered in this scheme.[24,25]

Provided that k_{des1} and k_{des2} are similar, scheme 2 dictates that at noninhibiting concentrations, partial and efficacious agonists induce nAcChoR desensitization *via* different pathways; efficacious agonists induce desensitization primarily through the open state ($A_2O \rightarrow D$), whereas partial agonists do so primarily through the preopen state ($A_2R \rightarrow D$). Consequently, efficacious agonists desensitize nAcChoRs with an apparent affinity that is strongly dependent on the value of K_o, whereas partial agonists do not. We reasoned that this critical difference in desensitization kinetics could be exploited to determine whether isoflurane increases the nAcChoR's apparent agonist affinity primarily by reducing the nAcChoR's agonist dissociation constant or, alternatively, by reducing the equilibrium constant between the preopen and open channel states.

In this study, we first validated scheme 2 for describing the kinetics of nAcChoR desensitization induced by the very efficacious agonist acetylcholine (K_o = 0.039) and the weak partial agonist suberyldicholine (K_o = 19.1) over a range of agonist concentrations that spans five orders of magnitude.[19,26] We confirmed that k_{des1} and k_{des2} are similar but found that k_{des3} is approximately twofold to fourfold faster. We then analyzed the effects of isoflurane on the kinetics of acetylcholine- and suberyldicholine-induced desensitization within the context of this scheme. We determined that over a wide range of agonist concentrations, a reduction in the value of K_{d2}, the microscopic dissociation constant for agonist binding to the lower affinity resting state site, primarily accounts for the effect of isoflurane on the apparent rates of nAcChoR desensitization.

Materials and Methods

Materials

Torpedo nobiliana was obtained from Biofish Associates (Georgetown, MA), and diisopropylfluorophosphate, acetylcholine, and suberyldicholine were obtained from Sigma Chemical Co. (St. Louis, MO). The fluorescent agonist, [1-(5-dimethylaminonaphthalene-)sulfonamido] *n*-hexanoic acid β-(*N*-trimethylammonium bromide) ethyl ester (Dns-C_6-Cho), was synthesized according to the procedure of Waksman *et al.*[27] Isoflurane was purchased from Anaquest (Murray Hill, NJ).

Methods

Preparation, Characterization, and Isoflurane Exposure of nAcChoR-rich Membranes. Receptor membranes were obtained from *Torpedo nobiliana* electric organs and purified by sucrose density gradient centrifugation essentially as described by Braswell *et al.*[28] Membranes were stored in *Torpedo* physiologic solution (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM $MgCl_2$, 5 mM NaH_2PO_4, and 0.02% NaN_3, pH 7.0) at −80°C and thawed on the day of use. Acetylcholinesterase activity was inhibited by exposing membranes to 3.0 mM diisopropylfluorophosphate for 30 min before dilution with *Torpedo* physiologic solution to obtain the desired receptor concentration. The number of agonist binding sites was determined from Dns-C_6-Cho titrations as previously described.[29] Solutions containing isoflurane were prepared from saturated *Torpedo* physiologic solutions at room temperature assuming a saturated solubility of 15 mM.[30] All kinetic experiments were performed at 20 ± 0.3°C.

Determination of the Dissociation Constant Defining Dns-C_6-Cho Binding to the nAcChoR's Agonist Self-inhibitory Site. Receptor-rich membranes were loaded into one of the stopped-flow spectrofluorometer syringes (Applied Photophysics, Leatherhead, United Kingdom), and 10 mM acetylcholine along with Dns-C_6-Cho (2–120 μM) were loaded into the other. Where appropriate, all solutions also contained isoflurane at the desired concentration. The two syringes were rapidly mixed (1 ms mixing time; 1:1 vol:vol) and the fluorescence intensity was recorded for 1 s. The excitation wavelength was 290 nm, and the monochromator bandpass was 5 nm. Fluorescence emission > 500 nm was measured through a high pass filter. In a typical experiment, three individual runs were signal averaged to reduce noise. In our experiments, the rapid addition of 5 mM acetylcholine (final concentration) saturates the two agonist binding sites on resting state nAcChoRs, leading to channel opening. Dns-C_6-Cho, a fluorescent agonist that has a relatively high affinity for the agonist self-inhibitory site, then binds to the self-inhibitory site on the open channel state and induces a receptor conformational transition.[25,27] This conformational transition produces an approximately exponential fluorescence enhancement whose rate was determined by fitting the recorded fluorescence trace to an exponential equation along with a linear component.[12] The linear component of the traces represented < 10% of the total amplitude and was not analyzed in detail. The observed rate of this fluorescence enhancement, k_{obs}, varies with Dns-C_6-Cho concentration according to the following equation[25]:

$$k_{obs} = \frac{k_f}{1 + K_B/[\text{Dns} - \text{C}_6 - \text{Cho}]} + k_b \qquad (1)$$

where K_B is the Dns-C_6-Cho dissociation constant for the agonist self-inhibitory site, and k_f and k_b are the forward and backward rate constants, respectively, describing the conformational transition that is induced by Dns-C_6-Cho binding to this site.

Determination of the Apparent Rate of Agonist-induced Desensitization. The apparent rate of agonist-induced desensitization was determined with a double agonist pulse assay using the stopped-flow spectrofluorometer in the sequential mixing configuration as previously described.[12] This assay permits the measurement of desensitization rates even in the presence of isoflurane, an anesthetic whose predominant effect is to block ion flux. Briefly, receptor membranes were loaded into one of the spectrofluorometer's premix syringes, and agonist was loaded into the other premix syringe. The solutions were rapidly mixed (1:1 vol:vol) and allowed to preincubate for the desired time. The number of nondesensitized receptors that remained after preincubation with agonist was quantitated from the amplitude of the fluorescence enhancement observed when the nAcChoR/agonist solution is mixed with 10 μM Dns-C_6-Cho and sufficient acetylcholine to open all remaining resting state nAcChoRs (5 mM). The apparent rate of desensitization was determined from an exponential fit of a plot of the fluorescence amplitude *versus* preincubation time. At least 10 preincubation time points were used for the determination of each apparent rate. Where appropriate, all solutions also contained isoflurane at the desired concentration. The excitation wavelength was

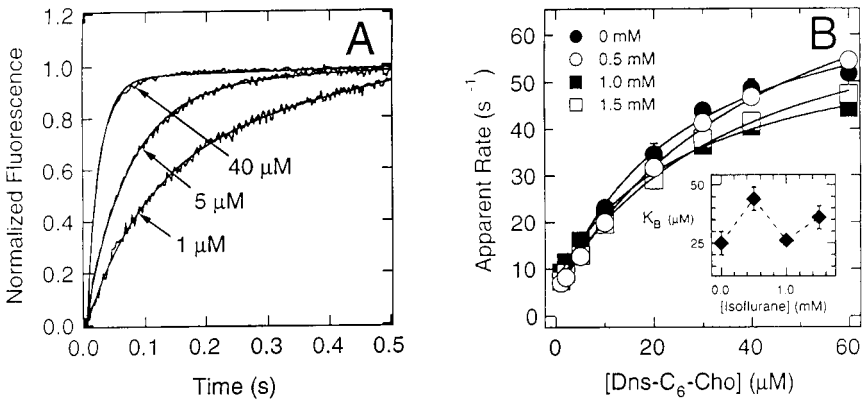


Fig. 1 (*A*) The fluorescence enhancement recorded on rapidly mixing nAcChoR-rich membranes with 5 mM acetylcholine and either 1 μM, 5 μM, or 40 μM Dns-C_6-Cho (final concentrations). The curve superimposed over each trace is a fit of the data to an exponential equation with a linear component. The rates of the fluorescence enhancement induced by 1 μM, 5 μM, and 40 μM Dns-C_6-Cho were 9.4 ± 0.3 s^{-1}, 14.1 ± 0.2 s^{-1}, and 46.9 ± 0.5 s^{-1}, respectively. (*B*) The rate of the fluorescence enhancement is plotted as a function of Dns-C_6-Cho concentration in the absence of isoflurane (0 mM) and in the presence of 0.5 mM, 1.0 mM, or 1.5 mM isoflurane. The curve is a fit of the data to equation 1. (*Inset*) The K_d of Dns-C_6-Cho for the nAcChoR agonist self-inhibitory site (K_B) is plotted as a function of isoflurane concentration.

Table 1. Effect of Isoflurane on Dns-C_6-Cho Binding to the Agonist Self-inhibition Site

Kinetic Constants in Equation 1	Control	0.5 mM Isoflurane	1.0 mM Isoflurane	1.5 mM Isoflurane
K_B	26 ± 5 μM	44 ± 5 μM	26 ± 2 μM	37 ± 5 μM
k_f	70 ± 12 s^{-1}	88 ± 5 s^{-1}	54 ± 1 s^{-1}	68 ± 4 s^{-1}
k_b	4 ± 1 s^{-1}	4 ± 1 s^{-1}	7.4 ± 0.4 s^{-1}	5.1 ± 0.8 s^{-1}

290 nm, and the monochromator bandpass was 5 nm. Fluorescence emission > 500 nm was measured through a high pass filter. Fluorescence intensity was recorded for 500 ms after the second mixing step. In a typical experiment, three to five individual runs were signal averaged to reduce noise.

Statistical Analysis and Curve Fitting. Data points on all figures represent the average of at least three determinations, and the error bars indicate the SD. Where the data points are larger than the errors, the error bars have been omitted. Data were fit with the analysis program Igor 3.01 (Wavemetrics, Lake Oswego, OR). The reported errors for the fitted parameters are the SDs derived from iterative curve fits.

Results

Effect of Isoflurane on the Binding of the Fluorescent Agonist Dns-C_6-Cho to the Agonist Self-inhibitory Site

Figure 1A shows representative fluorescence traces recorded when receptor-rich membranes were rapidly mixed with 5 mM acetylcholine and either 1 μM, 5 μM, or 40 μM Dns-C_6-Cho (final concentrations). For clarity, the traces have been normalized to the same maximal fluorescence amplitude. In this experiment, the rates of the fluorescence enhancement in the presence of 1 μM, 5 μM, and 40 μM Dns-C_6-Cho were 9.4 ± 0.3 s^{-1}, 14.1 ± 0.2 s^{-1}, and 46.9 ± 0.5 s^{-1}, respectively. Figure 1B plots the rate of the fluorescence enhancement *versus* Dns-C_6-Cho concentration in the absence of isoflurane and in the presence of 0.5 mM, 1 mM, or 1.5 mM isoflurane. At all Dns-C_6-Cho concentrations, the rate of the fluorescence enhancement in the presence of isoflurane was within 29% of the control rate obtained in the absence of isoflurane. The dissociation constant defining Dns-C_6-Cho binding to the agonist self-inhibition site, K_B, was determined to be 26 ± 5 μM in the absence of isoflurane from a fit of the control data in figure 1B to equation 1. The value of K_B did not vary systematically with isoflurane concentration and was 44 ± 5 μM, 26 ± 2 μM, and 36 ± 5 μM in the presence of 0.5 mM, 1.0 mM, and 1.5 mM isoflurane, respectively (fig. 1B, inset; table 1).

Agonist Concentration–Dependence of the Apparent Rate of Desensitization

Figure 2A plots the apparent rate of nAcChoR desensitization as a function of acetylcholine concentration. Over the concentration range shown (1 μM to 1 mM), acetylcholine opens channels but does not inhibit ion flux.[20] The apparent rate of desensitization increased with acetylcholine concentration before reaching a pla-

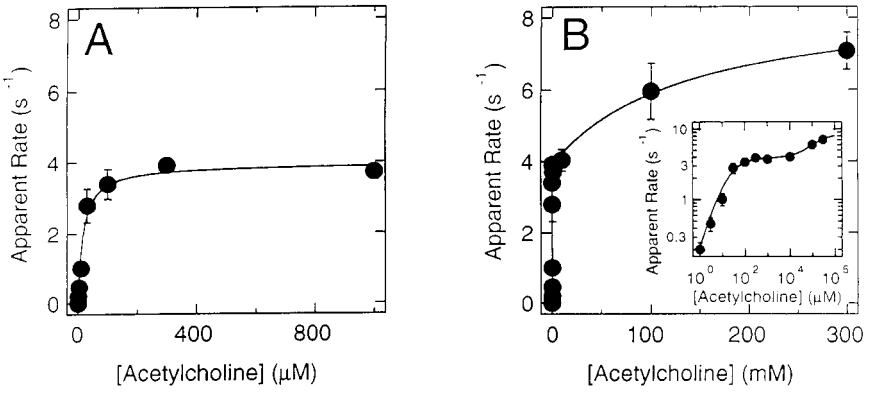


Fig. 2. The apparent rates of desensitization as a function of acetylcholine concentration. (*A*) The apparent rates induced by channel-opening acetylcholine concentrations are shown. (*B*) The apparent rates induced by acetylcholine concentrations that induce agonist self-inhibition are emphasized. (*Inset*) The data in (*A*) and (*B*) are presented on a double logarithmic plot to facilitate inspection of the acetylcholine concentration–dependence of the apparent rate of desensitization over an acetylcholine concentration range that induces both channel opening and inhibition. The curve was obtained from a fit of the data to equation 3 assuming a value of 0.039 for K_o as previously reported.[19] The values of the kinetic constants derived from this fit are given in table 2.

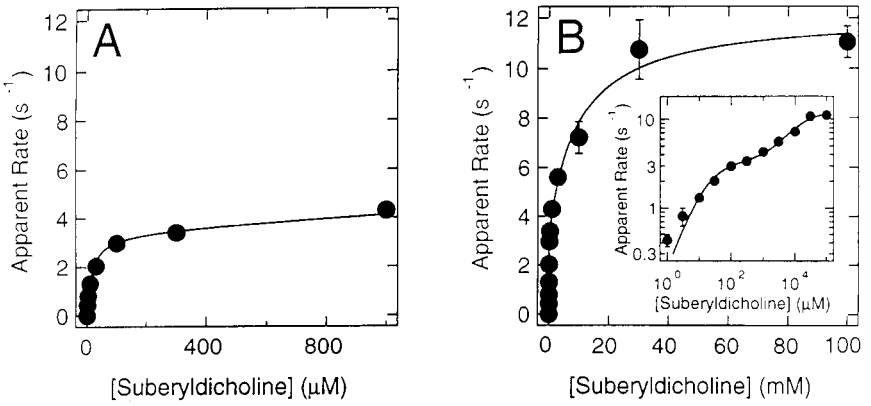


Fig. 3. The apparent rates of desensitization as a function of suberyldicholine concentration. (*A*) The apparent rates induced by channel-opening suberyldicholine concentrations are shown. (*B*) The apparent rates induced by self-inhibiting suberyldicholine concentrations are emphasized. (*Inset*) The data in (*A*) and (*B*) are presented on a log–log plot. The curve was obtained from a fit of the data to equation 3 assuming a value of 19.1 for K_o as previously reported.[26] The values of the kinetic constants derived from this fit are given in table 2.

teau of 3 s^{-1} by 100 μM. At much higher, self-inhibiting concentrations of acetylcholine (> 30 mM), the apparent rate of desensitization increased further, reaching 7.1 ± 0.5 s^{-1} by 300 mM acetylcholine (fig. 2B). Figures 3A and 3B plot the apparent rate of nAcChoR desensitization as a function of suberyldicholine concentration using the same receptor preparation that was used in the acetylcholine experiments shown in figures 2A and 2B. The same membrane preparation was used in figures 2 and 3 to compare more easily acetylcholine- and suberyldicholine-induced desensitization kinetics without the potentially confounding effects of variability between membrane preparations. Figure 3A plots the apparent rate of desensitization as a function of suberyldicholine concentration over a concentration range that opens channels but does not inhibit them.[20] The apparent rate of desensitization increased with suberyldicholine concentration before reaching a plateau of 3–4 s^{-1} by 100 μM. At the high, self-inhibiting suberyldicholine concentrations emphasized in figure 3B (> 1 mM), the apparent rate of desensitization increased further to 11 ± 0.6 s^{-1} by 100 mM.

Analysis of the Agonist Concentration–Dependence of the Apparent Rates of Desensitization within the Context of Scheme 2

Scheme 2 predicts that the apparent rate of desensitization will vary with agonist concentration as follows (see appendix):

$$^{app}k_{des} = \frac{k_{des1}K_o + k_{des2} + k_{des3}[A]/K_B}{\frac{K_oK_{d2}}{[A]^2}(K_{d1} + [A]) + K_o + 1 + ([A]/K_B)} \quad (2)$$

Agonist binding and electrophysiologic studies have shown that the value of K_{d1} is 100–1,000-fold lower than that of K_{d2} in *Torpedo* nAcChoRs.[25,31,32] Therefore, although the apparent rate of desensitization is highly dependent on K_{d2}, it is essentially independent of K_{d1}; for all agonist concentrations that are sufficiently high to produce appreciable binding to the lower affinity site, the higher affinity sites on resting state nAcChoRs will be nearly saturated with agonist. This explains our previous observation that the Hill coefficients for acetylcholine and carbamylcholine concentration–response curves for desensitization are near 1 rather than 2.[12] In addition, because the maximal (plateau) apparent rates of desensitization induced by suberyldicholine and acetylcholine at noninhibiting concentrations are similar (3–4 s^{-1}) in the absence of isoflurane and well as in its presence (see next section), the rate constants for desensitization *via* the preopen and open states (k_{des1} and k_{des2}, respectively) are assumed to be similar in the absence of isoflurane and well as in its presence. Therefore, for our desensitization experiments, equation 2 may be closely approximated as:

$$^{app}k_{des} = \frac{k_{des12}K_o + k_{des12} + k_{des3}[A]/K_B}{(K_oK_{d2}/[A]) + K_o + 1 + ([A]/K_B)} \quad (3)$$

where the rate constants for desensitization from the preopen and open states are assumed to be equivalent and equal to k_{des12}.

Previous ion flux studies using *Torpedo* nAcChoRs in native membranes have determined that in the absence of anesthetic, K_o values for acetylcholine and suberyldicholine are 0.039 and 19.1, respectively.[19,26] The values of the remaining four kinetic constants were determined by iterative curve fitting of the biphasic data in figures 2 and 3 to equation 3 while keeping K_o constant and equal to 0.039 (for acetylcholine) or 19.1 (for suberyldicholine). The values of the kinetic constants derived from

Table 2. Kinetic Parameters in Equation 3 Derived from Plots of the Apparent Rate of Desensitization *versus* Agonist Concentration and Comparison Values Obtained Using Ion Flux Techniques

Kinetic Constants in Equation 3	Acetylcholine		Suberyldicholine	
	Desensitization	Ion Flux	Desensitization	Ion Flux
K_o	0.039*	0.039*	19.1†	19.1†
K_2	450 ± 80 μM	972 ± 53 μM†	16 ± 7 μM	11.1 ± 1.6 μM*
K_B	130 ± 70 mM	40 mM‡	0.5 ± 0.1 mM	0.7 ± 0.11mM*
k_{des12}	3.9 ± 0.1 s^{-1}	6.7 ± 0.12 s^{-1}*	3.3 ± 0.3 s^{-1}	—
k_{des3}	8.5 ± 0.9 s^{-1}	—	12.1 ± 0.5 s^{-1}	—

* From reference 19.

† From reference 26.

‡ From reference 21.

these fits are given in table 2. For comparison, table 2 also gives their values as previously determined using ion flux techniques assuming a similar kinetic model. The nearly complete overlap of the experimental data points by the curve fits to equation 3 in figures 2 and 3 and the close agreement between the binding constants determined using the analysis of desensitization kinetics and those previously determined using ion flux techniques confirms the validity of scheme 2 and equation 3 for describing the kinetics of agonist binding, channel opening, and desensitization.

Analysis of the Effect of Isoflurane on the Apparent Rates of Desensitization within the Context of Scheme 2

In a previous study, we analyzed the effect of isoflurane on acetylcholine-induced desensitization kinetics and determined that isoflurane increases the nAcChoR's apparent affinity for acetylcholine.[12] This is reflected in figure 4A as an isoflurane-induced leftward shift in the concentration–response curve at noninhibiting acetylcholine concentrations. At a concentration of 1 mM, isoflurane reduced the nAcChoR's apparent dissociation constant for acetylcholine (defined as the acetylcholine concentration that induces a half-maximal apparent desensitization rate) by 12-fold from 44 ± 4 μM to 3.6 ± 0.6 μM. By fitting the control data in figure 4A to equation 3 with K_o held constant at 0.039, we determined the equilibrium and rate constants for this receptor preparation in the absence of anesthetic and report the results in the figure legend (dotted curve). Because we did not use acetylcholine at concentrations that are sufficiently high to inhibit channels in that study, we have taken the values for K_B and k_{des3} from table 2, which were obtained using another preparation. The solid and dashed curves in figure 4 demonstrate the effect of reducing either K_{d2} and K_o by 12-fold. The complete overlap of the solid and dashed curves demonstrates that a 12-fold reduction in either K_{d2} or K_o can equally well account for the 12-fold increase in the nAcChoR's apparent affinity for acetylcholine. Smaller reductions in both K_{d2} and K_o could similarly account for the increase in apparent

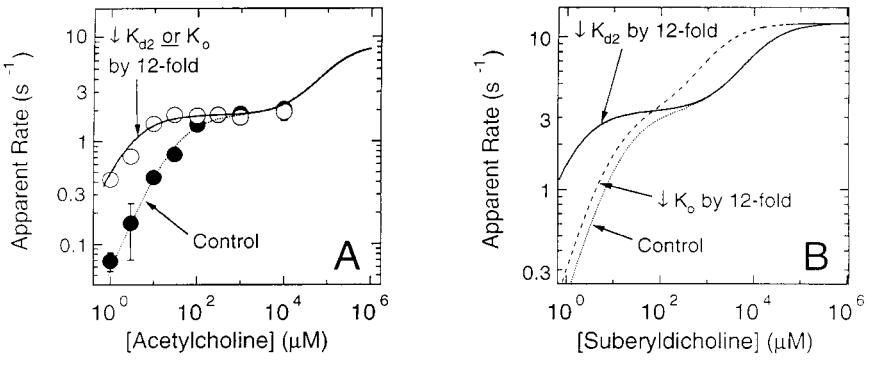


Fig. 4. (*A*) The apparent rate of desensitization is plotted as a function of acetylcholine concentration in the absence of isoflurane (filled circles) and in the presence of 1.0 mM isoflurane (open circles) from reference 12. The dotted curve is a fit of the control data (no isoflurane) to equation 3 assuming a value of 0.039 for K_o.[19] Because only channel-opening concentrations of acetylcholine were used in that study, the values for K_B and K_{des3} were taken from table 2 using another preparation. The completely overlapping solid and dashed curves demonstrate the effect of reducing either K_{d2} or K_o by 12-fold. (*B*) The apparent rate of desensitization *versus* suberyldicholine concentration determined using equation 3 is plotted. The dotted curve is derived from the kinetic constants in table 2 (for suberyldicholine) in the absence of isoflurane, whereas the solid and dashed curves demonstrate the effect of reducing either K_{d2} or K_o by 12-fold.

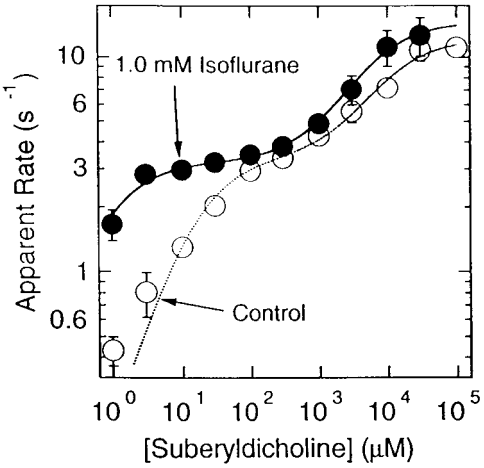


Fig. 5. The apparent rate of desensitization as a function of suberyldicholine concentration in the presence of 1 mM isoflurane and in the absence of anesthetic (control). The dotted curve is a fit of the control data (no isoflurane) to equation 3 with K_o equal to 19.1.[26] The solid curve is a fit of the 1.0-mM isoflurane data to equation 3 assuming no change in K_B. The kinetic constants derived from the fit is given in table 3.

affinity, indicating that there is no unique solution to equation 3 when the very efficacious agonist acetylcholine is used as the desensitizing agonist (curves not shown). Conversely, equation 3 predicts that equivalent reductions in K_{d2} and K_o will have dramatically different effects on the agonist concentration–dependence of the apparent rate of desensitization when using the weak partial agonist suberyldicholine (fig. 4B). Specifically, equation 3 predicts that at channel-opening suberyldicholine concentrations, an isoflurane-induced reduction in K_{d2} will cause a relatively larger increase in the apparent desensitization rate than an identical reduction in K_o, whereas at channel-inhibiting suberyldicholine concentrations, the reverse is true. The significance of this observation is that it implies that the effects of isoflurane on K_{d2} and K_o may be distinguished by analyzing the kinetics of nAcChoR desensitization induced by suberyldicholine over a wide concentration range.

Figure 5 plots the apparent rate of desensitization as a function of suberyldicholine concentration in the presence of 1.0 mM isoflurane along with the control rates obtained in the same preparation in the absence of anesthetic. Isoflurane substantially increased the apparent rates of desensitization induced by low concentrations of suberyldicholine but had a relatively small effect on that induced by high concentrations. The values of the rate constants in the presence of 1.0 mM isoflurane were determined by iterative curve fitting of the data in figure 5 to equation 3. Because isoflurane concentrations even as high as 1.5 mM did not significantly affect the binding of the fluorescent agonist Dns-C_6-Cho to the agonist self-inhibitory site on the nAcChoR (fig. 1B), we concluded that isoflurane does not significantly change K_B from its control value determined in the absence of anesthetic. Therefore, K_B was held constant and equal to 0.5 mM for this membrane preparation, and the values of the remaining four kinetic constants were permitted to vary. The results of this fit are plotted in figure 5A, and the kinetic constants are given in table 3 (preparation 2). This analysis indicated that the kinetic constant most affected by isoflurane was K_{d2}, which was reduced by more than an order of magnitude from 16 ± 7 μM to 0.9 ± 0.3 μM, whereas K_o was reduced only in half from 19.1 to 10 ± 1. The rate constants for desensitization, k_{des12} and k_{des3}, remained essentially unchanged.

Using two other receptor preparations, we also examined the effects of 0.5 and 1.5 mM isoflurane on the kinetics of suberyldicholine-induced desensitization (fig. 6 and table 3). Using the approach previously detailed, we determined that 0.5 mM isoflurane reduced K_{d2} from 18 ± 9 μM to 9 ± 2 μM, whereas K_o was reduced from 19.1 to 13 ± 1. At a concentration of 1.5 mM, isoflurane reduced K_{d2} by 34-fold from 17 ± 5 μM to 0.5 ± 0.1 μM but reduced K_o only from 19.1 to 5 ± 1. The rate constants for desensitization were little affected by isoflurane in either of these receptor preparations.

Table 3. Effect of Isoflurane on the Kinetic Parameters in Equation 3 for Suberyldicholine

Kinetic Constants in Equation 3	Receptor Preparation 1		Receptor Preparation 2		Receptor Preparation 3	
	Control	0.5 mM Isoflurane	Control	1.0 mM Isoflurane	Control	1.5 mM Isoflurane
K_o	19.1*	13 ± 1	19.1*	10 ± 1	19.1*	5 ± 1
K_2	18 ± 9 μM	9 ± 2 μM	16 ± 7 μM	0.9 ± 0.3 μM	17 ± 5 μM	0.5 ± 0.2 μM
K_B	0.23 ± 0.06 mM	0.23 mM	0.5 ± 0.1 mM	0.5 mM	0.2 ± 0.02 mM	0.2 mM
k_{des12}	2.2 ± 0.3 s^{-1}	2.7 ± 0.3 s^{-1}	3.3 ± 0.3 s^{-1}	3.2 ± 0.2 s^{-1}	1.6 ± 0.1 s^{-1}	1.9 ± 0.2 s^{-1}
k_{des3}	7.9 ± 0.3 s^{-1}	12.8 ± 0.2 s^{-1}	12.1 ± 0.5 s^{-1}	14.5 ± 0.5 s^{-1}	7.2 ± 0.1 s^{-1}	5.2 ± 0.1

* From reference [26].

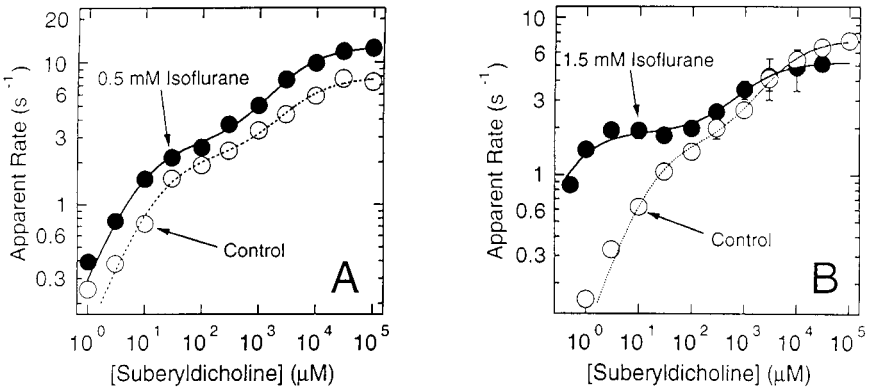


Fig. 6. (*A* and *B*) The apparent rate of desensitization is plotted as a function of suberyldicholine concentration in the presence of either 0.5 mM or 1.5 mM isoflurane, respectively. The dotted curves are fits of the control data (no isoflurane) to equation 3 with K_o equal to 19.1.[26] The solid curves are fits of the isoflurane data to equation 3 assuming no change in K_B. The kinetic constants derived from the fitting is given in table 3.

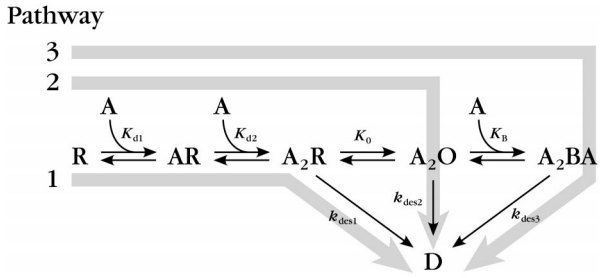
Discussion

Isoflurane Minimally Affects the Binding of a Fluorescent Agonist to the Agonist Self-inhibitory Site

In the presence of channel-opening concentrations of acetylcholine (5 mM), the fluorescent agonist Dns-C_6-Cho binds rapidly and with relatively high affinity to the nAcChoR's agonist self-inhibitory site. By measuring the Dns-C_6-Cho concentration–dependence of the observed rate of the resulting fluorescence enhancement, we determined that the dissociation constant of Dns-C_6-Cho for this site (K_B) is 26 ± 5 μM in the absence of isoflurane. As predicted by scheme 2, this value equals the concentration of Dns-C_6-Cho that is required to reduce by half the ion flux induced by maximally activating concentrations of acetylcholine (24 μM).[33] In the presence of isoflurane, the observed rates of the fluorescence enhancement, K_B, k_f, or k_b did not change systematically with isoflurane concentration. Consequently, we conclude that isoflurane has no significant effect on the kinetics of Dns-C_6-Cho binding to the agonist self-inhibitory site. Furthermore, because we used isoflurane at concentrations that also inhibit ion flux in this study, the lack of any significant effect by isoflurane on the binding of Dns-C_6-Cho to the agonist self-inhibition site strongly suggests that isoflurane and agonists (at self-inhibiting concentrations) inhibit ion flux by binding to distinct sites.[34]

Agonist Concentration–Dependence of the Apparent Rates of Desensitization and the Effect of Isoflurane

In our desensitization studies, we elected to use two agonists whose efficacies are markedly different; 96% of all receptors that bind two acetylcholine molecules open, whereas only 5% of receptors that bind two suberyldicholine molecules open. In addition, and importantly, self-inhibition is induced by both of these agonists only at concentrations that far exceed those required to open channels (in the absence of a transmembrane potential).[20] These features allow us to readily interpret our desensitization data in terms of distinct kinetic pathways in scheme 2:



At the noninhibiting concentrations shown in figures 2A and 3A, an agonist may induce desensitization *via* pathways 1 and/or 2. For a given agonist, the principal kinetic pathway leading to the desensitized state is dependent on K_o and the relative values of k_{des1} and k_{des2}. In *Torpedo* nAcChoRs, ion flux studies suggest that these two rate constants are not significantly different. Conversely, in mouse muscle nAcChoRs, $k_{des1} << k_{des2}$ as almost all desensitization proceeds through the open state.[35] Our observation that the apparent rates of desensitization induced by noninhibiting concentrations of acetylcholine and suberyldicholine increase and then plateau at similar values of 3–4 s^{-1} supports the conclusion that k_{des1} and k_{des2} are equal in nAcChoRs derived from *Torpedo.* Thus, our kinetic model dictates that suberyldicholine will desensitize 95% of all receptors from the preopen state (pathway 1), whereas acetylcholine will desensitize 96% from the open state (pathway 2).

At the higher, self-inhibiting concentrations emphasized in figures 2B and 3B, both suberyldicholine and acetylcholine desensitize receptors from the self-inhibited state (pathway 3).[20] Ion flux studies indicate that the apparent affinity of acetylcholine and suberyldicholine for the self-inhibitory site is on the order of 100 mM and 10 mM, respectively, in the absence of a transmembrane potential.[20] At these agonist concentrations, we consistently observed that the apparent rate of desensitization induced by either suberyldicholine or acetylcholine increased beyond the plateau rate observed at noninhibiting agonist concentrations, indicating that in scheme 2, k_{des3} is greater than either k_{des1} or k_{des2}.

Having established that scheme 2 can account for both ion flux and desensitization kinetics induced by a weak partial agonist and a very efficacious agonist over a very wide range of concentrations in the absence of anesthetic, we examined the effect of isoflurane on the agonist concentration–dependence of the apparent rate of desensitization within the context of that scheme. We demonstrated that our previously reported isoflurane-induced reduction in the nAcChoR's apparent acetylcholine dissociation constant may be modeled as a quantitatively identical reduction in either K_{d2} or K_o or smaller reductions in the values of both.[12] This occurs because for very efficacious agonists, the apparent agonist dissociation constant for desensitization (*via* the open state) approximates the product of K_{d2} and K_o. Conversely, because suberyldicholine desensitizes nearly all nAcChoRs without ever opening them, the apparent agonist dissociation constant for desensitization (*via* the preopen state) is relatively insensitive to changes in K_o and approximates K_{d2}. A fit of our 1 mM isoflurane data in figure 5 to equation 3 revealed that K_{d2} is reduced by 18-fold, whereas K_o is reduced only in half. Thus, within the context of scheme 2, the increase in the nAcChoR's apparent affinity for suberyldicholine is explained almost entirely as an isoflurane-induced reduction in the agonist dissociation constant. By studying a range of isoflurane concentrations, it was evident that K_{d2} decreases with isoflurane concentration, consistent with our previous observation that isoflurane increases the apparent agonist affinity in a concentration-dependent manner.[12]

Dilger *et al.* have used electrophysiologic techniques to define the effects of isoflurane on acetylcholine-activated ion flux through nAcChoRs expressed in BC3H-1 cells.[36] They also observed that isoflurane increases the nAcChoR's apparent agonist affinity.[36] From measurements of macroscopic currents induced by the very rapid application of agonist and the analysis of single channel data, they tentatively concluded that this increase in apparent agonist affinity reflected a reduction in the agonist dissociation constant rather than a change in channel gating kinetics.[36,37] Using a completely different approach, we have confirmed their tentative conclusion that isoflurane increases the apparent agonist affinity of the *Torpedo* nAcChoR primarily by reducing the agonist dissociation constant. Although the specific kinetic step altered by isoflurane has not yet been elucidated in studies of the $GABA_A$ receptor, a preliminary study by Li *et al.* suggested that halothane reduces the rate at which GABA dissociates from it receptor binding site.[38] Thus, volatile anesthetics may act similarly on both the nAcChoR and the $GABA_A$ receptor by altering agonist binding kinetics.

Appendix

The general approach for deriving the agonist concentration–dependence of the apparent desensitization rate has been previously reported.[19] According to scheme 2, the rate of formation of desensitized receptors is equal to the sum of the rates of desensitization from the A_2R, A_2O, and A_2DA states:

$$-\frac{d[D]}{dt} = k_{des1}[A_2R] + k_{des2}[A_2O] + k_{des3}[A_2BA] \qquad (A1)$$

The equilibrium constants in scheme 2 are defined as:

$$K_{d1} = \frac{[A][R]}{[AR]} \quad K_{d2} = \frac{[A][AR]}{[A_2R]} \quad K_o = \frac{[A_2R]}{[A_2O]} \quad K_B = \frac{[A][A_2O]}{[A_2BA]}$$

Substituting the equilibrium constants into A1 yields:

$$\begin{aligned} -\frac{d[D]}{dt} &= \frac{k_{des1}[A][AR]}{K_{d2}} + \frac{k_{des2}[A][AR]}{K_oK_{d2}} + \frac{k_{des3}[A]^2[AR]}{K_oK_{d2}K_B} \\ &= [AR]\left(\frac{k_{des1}[A]}{K_{d2}} + \frac{k_{des2}[A]}{K_oK_{d2}} + \frac{k_{des3}[A]^2}{K_oK_{d2}K_B}\right) \\ &= \frac{[AR]}{K_oK_{d2}[A]}\left(k_{des1}K_o[A]^2 + k_{des2}[A]^2 + \frac{k_{des3}[A]^3}{K_B}\right) \qquad (A2) \end{aligned}$$

The total receptor concentration equals the sum of the concentrations of all of the individual receptor conformational states

$$[R_T] = [R] + [AR] + [A_2R] + [A_2O] + [A_2BA] + [D]$$

This may be rewritten as

$$\begin{aligned} \frac{[R_T] - [D]}{[AR]} &= \frac{[R]}{[AR]} + 1 + \frac{[A_2R]}{[AR]} + \frac{[A_2O]}{[AR]} + \frac{[A_2BA]}{[AR]} \\ &= \frac{K_{d1}}{[A]} + 1 + \frac{[A]}{K_{d2}} + \frac{[A]}{K_oK_{d2}} + \frac{[A]^2}{K_oK_{d2}K_B} \\ &= \frac{1}{[A]K_oK_{d2}}\left(K_{d1}K_oK_{d2} + K_oK_{d2}[A] + K_o[A]^2 + [A]^2 + \frac{[A]^3}{K_B}\right) \end{aligned}$$

By rearrangement:

$$[AR] = \frac{K_o K_{d2}[A]}{K_o K_{d2}[A] + K_o[A]^2 + [A]^2 + \frac{[A]^3}{K_B}} ([R_T] - [D]) \quad (A3)$$

Substituting equation A3 into A2 yields:

$$-\frac{d[D]}{dt} = \frac{k_{des1} K_o[A]^2 + k_{des2}[A]^2 + \frac{k_{des3}[A]^3}{K_B}}{K_o K_{d1} K_{d2} + K_o K_{d2}[A] + K_o[A]^2 + [A]^2 + \frac{[A]^3}{K_B}} ([R_T] - [D])$$

$$= \frac{k_{des1} K_o + k_{des2} + k_{des3}[A]/K_B}{\frac{K_o K_{d2}}{[A]^2}(K_{d1} + [A]) + K_o + 1 + \frac{[A]}{K_B}} ([R_T] - [D]) \quad (A4)$$

Under conditions of excess agonist over receptor sites, receptor desensitization is a pseudo–first-order process characterized by an apparent rate constant, ${}^{app}k_{des}$:

$$-\frac{d[D]}{dt} = {}^{app}k_{des}([R_T] - [D])$$

Rearrangement yields:

$${}^{app}k_{des} = \frac{-(d[D]/dt)}{[RT] - [D]} \quad (A5)$$

Substituting equation A4 into equation A5 yields equation 2 in the Results section.

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