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General Anesthetics Modify the Kinetics of Nicotinic Acetylcholine Receptor Desensitization at Clinically Relevant Concentrations

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Background: **General anesthetics are thought to induce anesthesia through their actions on ligand-gated ion channels. One such channel, the nicotinic acetylcholine receptor (nAcChoR), can be found in different subtypes in the central nervous system and at the periphery in the neuromuscular junction. The latter subtype of the nAcChoR is a useful model for examining interactions between general anesthetics and ligand-gated ion channels, because it can be isolated and purified in sufficient quantities to allow for biophysical and biochemical studies. This study examines the actions of general anesthetics on agonist-induced conversion of the nAcChoR to inactive desensitized conformational states.**

Methods: **Nicotinic acetylcholine receptor membranes were purified from the electric organ of *Torpedo nobiliana*. Agonist-induced desensitization was characterized from the time-dependent increase in fluorescence intensity that results from the binding of the fluorescent acetylcholine analog, Dns-C_6-Cho, to the nAcChoR.**

Results: **Mixing Dns-C_6-Cho with nAcChoR-rich membranes results in an increase in fluorescence that is characterized by four rate processes. Concentrations of isoflurane and butanol, which range from subclinical to toxic increase the rates of the third and fourth components of fluorescence, corresponding to fast and slow desensitization, respectively. At concentrations that are twice their EC_{50}s for anesthesia, isoflurane, butanol, chloroform, methanol, and cyclopentane-methanol increase the apparent rates of fast and slow desensitization by an average of 92 ± 22% and 108 ± 22%, respectively.**

Conclusions: **The concentration range over which general anesthetics modify the kinetics of nAcChoR desensitization is similar to those reported for anesthetic actions on the $GABA_A$ receptor. Thus, the nAcChoR, like other members of this superfamily, is a sensitive target of general anesthetics. (Key words: Alcohols. Anesthetics, volatile. Desensitization. Neuromuscular junction. Nicotinic acetylcholine receptor. Theories of anesthetic action.)**

This article is accompanied by a Highlight. Please see this issue of ANESTHESIOLOGY, page 31A.

ALTHOUGH anesthesia is generally thought to result from alterations in neuronal synaptic transmission, the mechanisms by which this occurs have not been fully defined. Progress in this regard has been made by studying the effects of anesthetics on well characterized membrane protein systems such as membranes containing the nicotinic acetylcholine receptor (nAcChoR). This receptor is one member of a superfamily of structurally related ligand-gated ion channels that includes the GABA, glycine, and NMDA receptors.[1] In contrast to other members of this superfamily, the nAcChoR can be isolated in quantities large enough to allow for spectroscopic and biochemical studies.[2,3] Consequently, the nAcChoR has become the best characterized ligand-gated ion channel. Because all members of this superfamily have four membrane-spanning domains and considerable amino acid sequence homology, conformational information obtained from studies using the nAcChoR is likely to be relevant to the other members.

Studies of nAcChoR conformational states using radioligand techniques indicate that, in the absence of agonist, the nAcChoR can be considered to exist in equilibrium between two conformations: an activatable

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resting state that binds agonist with low affinity and an inactive desensitized state that binds agonist with high affinity. Within the context of this model, approximately 80% of the nAcChoRs are in the resting state and 20% are in the desensitized state.[4,5] Such studies reveal that agonists induce a slow (seconds to minutes) conversion of low-affinity receptors to the high-affinity, desensitized state. This process generally is referred to as agonist-induced slow desensitization.

Using radioligand assays, several groups have demonstrated that general anesthetics increase the apparent affinity of agonists for the nAcChoR.[6–8] This has been interpreted to mean that anesthetics stabilize the receptor's high-affinity conformational state. Because this conformation is inactive, this interpretation has broad implications in terms of molecular mechanisms of anesthetic action, because it demonstrates how anesthetics can modulate the function of ligand-gated ion channels.

With the application of techniques having faster temporal resolution, it has been possible to obtain a more complete kinetic description of receptor conformational transitions.[9–12] Such methods have resolved an active conformational state and a fast phase of desensitization. This fast phase of desensitization reflects agonist binding to a desensitized conformational state that has an intermediate affinity for agonist (fast desensitized state).[11,13] The transition from the resting and/or active state to the fast desensitized state occurs over hundreds of milliseconds.[12,14] Because conformational transitions to the fast desensitized state occur within the time frame required to perform radioligand experiments, assumptions based on such techniques may not be valid. For example, it is apparent that the low-affinity state of the receptor detected using radioligand binding methods is the fast desensitized state and that the resting-state affinity for agonist is 100 times lower than the value reported in those studies. On the basis of kinetic studies having millisecond time resolution, an allosteric model has been developed that includes four discrete interconvertible conformational states (fig. 1).[11,13]

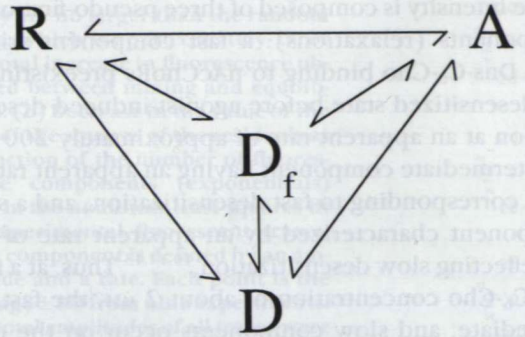

Fig. 1. Four-state allosteric model for nAcChoR conformational transitions. R = resting state; A = open (active) state; D_f = fast desensitized state; D = slow desensitized state.

We have characterized in detail the actions of two general anesthetics (isoflurane and butanol) on the kinetic processes of fast and slow desensitization as well as on the fraction of nAcChoRs preexisting in the high-affinity, slow desensitized state before agonist-induced desensitization using stopped-flow fluorescence spectroscopy. We observe that general anesthetics significantly enhance the rates of fast and slow desensitization. This action occurs at clinically relevant anesthetic concentrations. In contrast, alterations in the fraction of nAcChoRs preexisting in the slow desensitized state occur only at anesthetic concentrations that far exceed those required to induce general anesthesia.

Materials

Torpedo nobiliana was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate and (dansylaminoethyl) trimethylammonium perchlorate were purchased from Sigma Chemicals (St. Louis, MO). The fluorescent agonist, [1-(5-dimethylamino naphthalene)sulfonamido] n-hexanoic acid b-(N-trimethylammonium bromide) ethyl ester (Dns-C_6-Cho), was synthesized according to the procedure of Waksman *et al.* (fig. 2).[15] Isoflurane was purchased from Anaquest (Murray Hill, NJ). Butanol and cyclopentanemethanol were from Aldrich Chemical Co. (Milwaukee, WI), chloroform from American Scientific Products (McGraw Park, IL), and methanol from Fischer (Pittsburgh, PA). Gas chromatography was performed on a Hewlett Packard 5890 gas chromatograph equipped with a J and W (Folsom, CA) DB-WAX 122-7033 column.

Methods

Preparation and Characterization of nAcChoR Membranes

Electric organs of *T. nobiliana* were dissected, and membrane fragments were prepared by sucrose density gradient centrifugation at 4°C as previously described and approved by the Massachusetts General Hospital Animal Care and Use Committee.[16] Membranes were stored in Torpedo physiologic saline (250 mM NaCl, 5 mM KCl, 3 mM CaCl, 2 mM $MgCl_2$, 5 mM NaH_2PO_4,

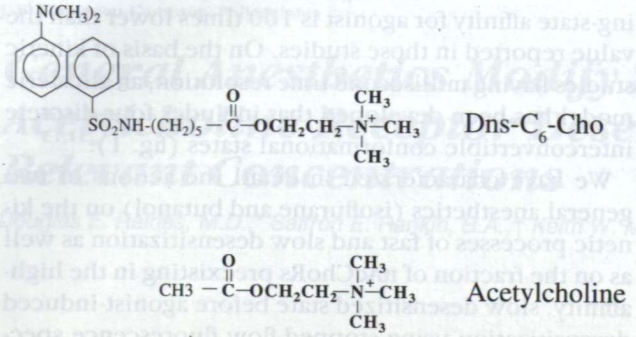

Fig. 2. Structures of the fluorescent agonist Dns-C_6-Cho and acetylcholine.

and 0.02% NaN_3, *p*H 7.0) at −80°C and used within 48 h of being thawed. The number of agonist binding sites was determined from acetylcholine competition of (dansylaminoethyl) trimethylammonium perchlorate binding as described by Neubig and Cohen.[17] Acetylcholinesterase activity was inhibited by exposing membrane fragments to 1.0 mM diisopropylfluorophosphate for 30 min. Fluorescence intensity was recorded with an SX.17 stopped-flow spectrofluorimeter (Applied Photophysics, Leatherhead, England) through a 560-nm high-pass filter (Omega Optical, Brattleboro, VT).

Stopped-Flow Fluorescence Spectroscopy

Membrane fragments containing the nAcChoR were mixed with Torpedo physiologic saline containing the appropriate concentration of the desired general anesthetic in a gas-tight syringe to achieve a receptor concentration of 0.8 μM in agonist binding sites. Solutions containing volatile anesthetics were prepared from dilutions of anesthetic-saturated Torpedo physiologic saline assuming saturated solubilities of 15 mM and 66.7 mM, respectively, for isoflurane and chloroform. Isoflurane and butanol were studied over a wide range of concentrations. Methanol, chloroform, and cyclopentanemethanol were each studied at a concentration equal to twice their EC_{50} for anesthesia. Membranes were equilibrated with the desired anesthetic at 20°C for 30–60 min. Gas chromatography revealed that, even with isoflurane, the most volatile anesthetic studied, evaporative loss of anesthetic during mixing and equilibration was negligible. Membranes were then rapidly mixed with an equal volume of 4.0 μM Dns-C_6-Cho (plus the desired anesthetic) in the stopped-flow spectrofluorimeter, yielding final concentrations of 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites. With this system, two solutions can be mixed within 1 ms. An excitation wavelength of 290 nm was provided by a 150-watt xenon arc lamp and monochromator. Fluorescence emission above 560 nm was measured. Fluorescence intensity was recorded (2,000 points over 100 s) on a logarithmic time base; each logarithmic unit of time (*i.e.*, 10–100 ms) contained 400 points. A logarithmic time base more evenly distributes data points among kinetic components occurring on different time scales than a simple linear time base. Individual shots were digitally stored. In a typical experiment, four to eight individual shots were signal-averaged to reduce noise. Signal-averaged fluorescent traces were transferred to a Macintosh Centris 650 and fit to the sum of exponentials using a nonlinear least squares algorithm with the commercially available analysis program Igor (Wavemetrics, Lake Oswego, OR). Details of this analysis are described below. Equilibration of membranes with anesthetics and data acquisition was performed at 20 ± 0.3°C. Data points on all figures represent the mean of at least three separate experiments. Error bars on data points indicate the standard deviations between experiments. Two fish prepared separately were used for these studies. Because no significant difference between fish was observed, the data were pooled. For convenience, the total increase in fluorescence intensity that occurs after mixing agonist with receptor membranes has been normalized to 1.0 in all figures.

Data Analysis

Rapid mixing of Dns-C_6-Cho with membranes containing the nAcChoR results in a time-dependent increase in fluorescence intensity (fig. 3).[10] Previous studies observed that, at a final concentration of approximately 2 μM Dns-C_6-Cho, this increase in fluorescence intensity is composed of three pseudo-first-order components (relaxations): a fast component arising from Dns-C_6-Cho binding to nAcChoRs preexisting in the desensitized state before agonist-induced desensitization at an apparent rate of approximately 200 s^{-1}, an intermediate component having an apparent rate of 1 s^{-1} corresponding to fast desensitization, and a slow component characterized by an apparent rate of 0.1 s^{-1} reflecting slow desensitization.[10,11,13] Thus, at a final Dns-C_6-Cho concentration of about 2 μM, the fast, intermediate, and slow components occur on the time scales of several milliseconds, hundreds of milliseconds, and seconds, respectively.

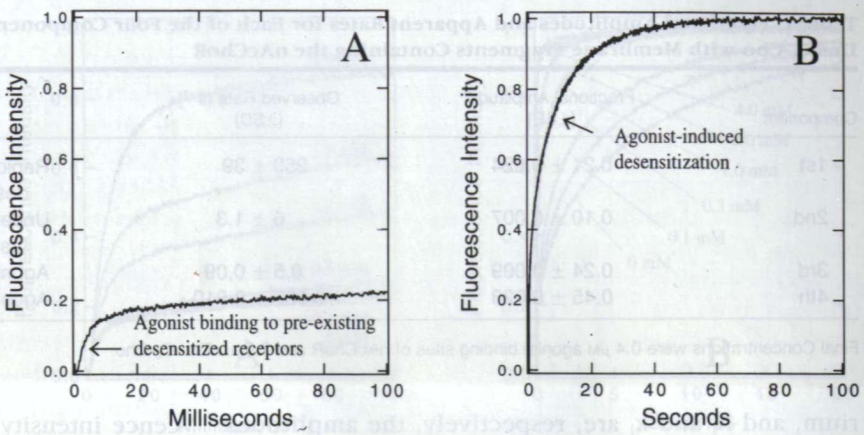

Fig. 3. Increase in fluorescence after mixing Dns-C_6-Cho with membranes containing the nAcChoR. Final concentrations were 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites. The same experiment is displayed in *A* and *B* but on different time scales. The component reflecting the binding of Dns-C_6-Cho to receptors in the high-affinity slow desensitized state (~20% of all receptors) occurs over 10–15 ms and is indicated by the arrow in *A*. The slower time scale in *B* demonstrates conversion of the low-affinity resting-state receptors to the high-affinity slow desensitized state (agonist-induced desensitization).

However, nonlinear least squares fit of our 2,000-point signal-averaged fluorescent traces to the sum of three (or fewer) exponentials had residuals that were clearly nonrandom (fig. 4A). We explored the possible existence of another small component, not resolved in earlier studies, by evaluating the improvement in fit obtained by allowing for additional components to fit the traces (fig. 4B). A logarithmic decrease in the sum of the squares of the residuals was obtained by using up to four exponentials to fit fluorescent traces obtained from control membranes (no anesthetic) or membranes that were equilibrated with physiologically relevant concentrations of anesthetics (*i.e.*, up to five times their EC_{50} for anesthesia in tadpoles). In either case, little decrease in the fitting error was obtained by using more than four exponentials. An approximate partial F test applied to the sum of the squares of the residuals indicated that fitting to an equation having four components significantly improved the fit over fitting to one having just three ($P < 0.001$).[18] As can be appreciated by examining figure 4A, the residuals resulting from fitting fluorescent traces to an equation having four exponentials, while not completely random, generally fall within the experimental noise. Therefore, the fluorescent traces were analyzed by iterative fitting to the equation

$$I_t = I_t - \sum_{i=1}^{4} A_i e^{-k_i t},$$

where I_t is the fluorescence emission at time t after mixing and I_e is the fluorescence emission at equilib-

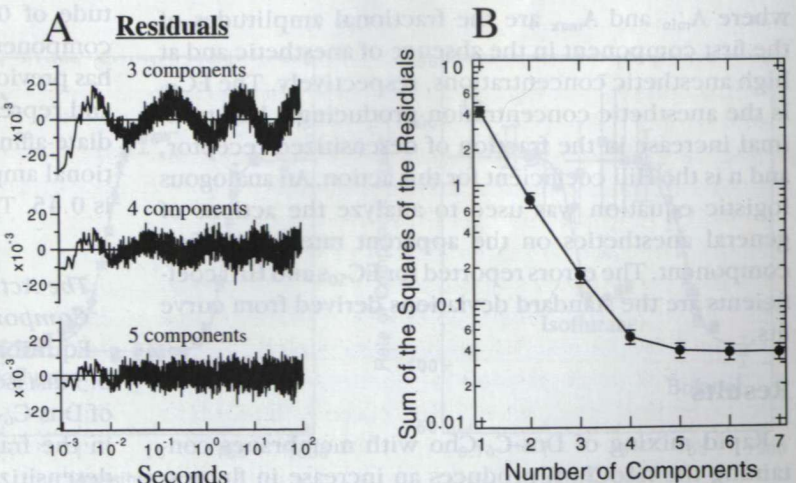

Fig. 4. (*A*) Residuals obtained from fitting the fluorescent trace from a typical experiment to three, four, or five components. When four components are used, the residuals generally are no larger than the random noise, which is approximately 1% of the total increase in fluorescence observed between mixing and equilibrium. (*B*) Decrease in the value of the sum of the squares of the residuals as a function of the number of fluorescence components (exponentials) used in the nonlinear least-squares fit of experimental fluorescent traces. Each component is defined by an amplitude and a rate. Each point is the average ± SD from nine experiments. The total amplitudes of all traces were normalized to 1.0. Final concentrations were 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites.

Table 1. Fractional Amplitudes and Apparent Rates for Each of the Four Components of Fluorescence Obtained after Mixing Dns-C_6-Cho with Membrane Fragments Containing the nAcChoR

Component	Fractional Amplitude (±SD)	Observed Rate (s^{-1}) (±SD)	Significance
1st	0.21 ± 0.024	250 ± 39	Rapid agonist binding to preexisting slow desensitized receptors
2nd	0.10 ± 0.007	6 ± 1.3	Undefined, possibility agonist binding to preexisting fast desensitized receptors
3rd	0.24 ± 0.009	0.5 ± 0.09	Agonist-induced fast desensitization
4th	0.45 ± 0.023	0.087 ± 0.010	Agonist-induced slow desensitization

Final Concentrations were 0.4 μM agonist binding sites of nAcChoR and 2.0 μM Dns-C_6-Cho.

rium, and A_i and k_i are, respectively, the amplitudes and rates of component i. A_{total} is equal to $A_1 + A_2 + A_3 + A_4$. The fractional amplitude of component i is defined as A_i/A_{total}.

These results suggest that the intermediate component reported by Heidmann and Changeux may be composed of at least two distinct components: a smaller one occurring in the 50–100-ms time scale and a larger one characterized by a time course of several hundred milliseconds (see Discussion). The larger component occurs on the time scale expected for Dns-C_6-Cho-induced fast desensitization of the nAcChoRs under the conditions used in this study (approximately 1 s).[10,11,13]

EC_{50}s and Hill coefficients for general anesthetic stabilization of the high-affinity state were determined by fitting a plot of the fractional amplitude of the first component *versus* anesthetic concentration to the following logistic equation:

$$\text{Fractional amplitude} = (A_{max} - A_{min}) \cdot \left(\frac{[\text{Anesthetic}]^n}{[\text{Anesthetic}]^n + EC_{50}{}^n}\right) + A_{min},$$

where A_{min} and A_{max} are the fractional amplitudes of the first component in the absence of anesthetic and at high anesthetic concentrations, respectively. The EC_{50} is the anesthetic concentration producing a half-maximal increase in the fraction of desensitized receptor, and n is the Hill coefficient for this action. An analogous logistic equation was used to analyze the actions of general anesthetics on the apparent rate of the first component. The errors reported for EC_{50}s and Hill coefficients are the standard deviations derived from curve fits.

Results

Rapid mixing of Dns-C_6-Cho with membranes containing the nAcChoR produces an increase in fluorescence intensity due to energy transfer from receptor tryptophans to the dansyl moiety of receptor-bound agonist.[10] Dns-C_6-Cho not bound to protein does not contribute significantly to the increase in fluorescence observed above 560 nm.[10] The fractional amplitudes and rates of each of the four components derived from nine separate experiments in the absence of anesthetic are presented in table 1. The fractional amplitude of the first component is 0.21. Because the agonist affinity of the slow desensitized state is much greater than that of all other conformational states (k_d = 3 nM), under our experimental conditions of excess Dns-C_6-Cho over agonist binding sites, essentially all receptors are converted to the slow desensitized state at equilibrium by the agonist.[10] Therefore, 0.21 equals the fraction of receptors preexisting in the slow desensitized state before agonist-induced fast and slow desensitization. This is consistent with previous radioligand and fluorescent agonist studies indicating that approximately 20% of nAcChoRs are in the high-affinity, desensitized state before agonist-induced desensitization.[4,5] The second component is the smallest, having a fractional amplitude of 0.10. The fractional amplitude of the third component is 0.24. It is the major component of what has previously been termed the "intermediate process" and represents receptor isomerization to the intermediate-affinity, fast desensitized state. Finally, the fractional amplitude of the fourth and slowest component is 0.45. This component reflects slow desensitization.

The Actions of General Anesthetics on the First Component of Fluorescence

Equilibration of nAcChoR membranes with less than 0.3 mM isoflurane or 36 mM butanol before the addition of Dns-C_6-Cho produces no more than a small increase in the fraction of nAcChoRs preexisting in the slow desensitized state, as reflected in a small increase in

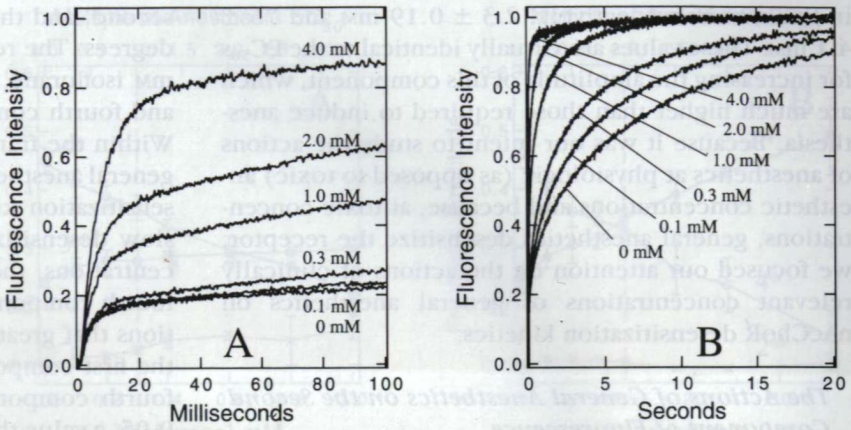

Fig. 5. Increase in fluorescence after mixing Dns-C_6-Cho with membranes containing the nAcChoR that were equilibrated with various concentrations of isoflurane for 30–60 min. Final concentrations were 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites. The same experiment is displayed in *A* and *B* but on different time scales. Relatively high concentrations of isoflurane increase the amplitude of the first component. Low concentrations have little action on this component. However, even subanesthetic concentrations of isoflurane can be seen to enhance agonist-induced desensitization in *B*.

the fractional amplitude of the first component (figs. 5 and 6A). At twice the EC_{50} for general anesthesia in tadpoles (0.6 mM isoflurane and 24 mM butanol), the amplitude of the first component of fluorescence is increased from 0.21 to 0.27 by isoflurane and is unchanged by butanol. At anesthetic concentrations that exceed those required to induce anesthesia, there is a substantial increase in the amplitude of this component reflecting an increase in the fraction of receptors preexisting in the desensitized state before agonist-induced desensitization. At near-saturating concentrations of either isoflurane or butanol, the fractional amplitude of the first component reaches a maximum of 0.75–0.80. For either anesthetic, a semilogarithmic plot of the amplitude of the first component *versus* anesthetic concentration is steeply sigmoidal (fig. 6). The respective EC_{50}s and Hill coefficients for this action are 2.3 ± 0.8 mM and 3.3 ± 0.36 for isoflurane and 80 ± 1.4 mM and 4.7 ± 0.34 for butanol. These concentrations of isoflurane and butanol are 7.7 and 6.7 times higher, respectively, than their EC_{50}s for inducing anesthesia in tadpoles.

At concentrations of isoflurane and butanol required to induce general anesthesia, there is no alteration in the rate of binding of Dns-C_6-Cho to nAcChoRs preexisting in the slow desensitized conformational state from its control value of 250 ± 39 s^{-1}. The apparent rate constant derived from this rate is 1.3 ± 0.20 × 10^8 $M^{-1} \cdot s^{-1}$, which is consistent with a process whose rate is limited by diffusion. At concentrations of anesthetic that induce the conversion of receptors from the resting to the slow desensitized state, this rate decreases (fig. 6B). At high anesthetic concentrations, this rate reaches a minimum of 155 ± 5.0 s^{-1} (0.78 ± 0.025 × 10^8 $M^{-1} \cdot s^{-1}$) for isoflurane and 110 ± 13 s^{-1} (0.55 ± 0.065 × 10^8 $M^{-1} \cdot s^{-1}$) for butanol. A fit of this data to a logistic equation reveals that the concentration of isoflurane or butanol required to cause a half-maximal decrease

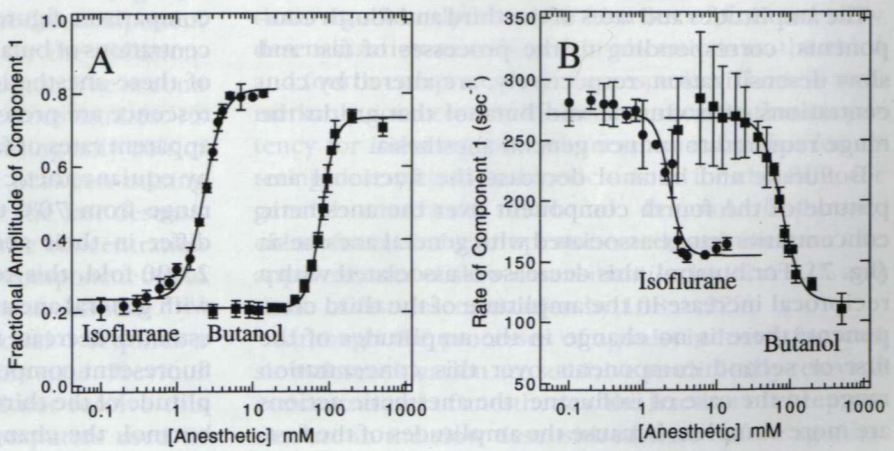

Fig. 6. Fractional amplitude (*A*) and apparent rate (*B*) of the first component after mixing Dns-C_6-Cho with membranes containing the AcChoR. Final concentrations were 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites. EC_{50}s for increasing the fractional amplitude of this component are 2.3 ± 0.8 mM and 80 ± 1.4 mM with Hill coefficients of 3.3 ± 0.36 and 4.7 ± 0.34 for isoflurane and butanol, respectively. (*B*) EC_{50}s for decreasing the apparent rate of this component are 2.3 ± 0.19 mM and 76 ± 4.1 mM with Hill coefficients of −5 ± 1.5 and −3.2 ± 0.50 for isoflurane and butanol, respectively.

in this rate is, respectively, 2.3 ± 0.19 mM and 76 ± 4.1 mM. These values are virtually identical to the EC_{50}s for increasing the amplitude of this component, which are much higher than those required to induce anesthesia. Because it was our intent to study the actions of anesthetics at physiologic (as opposed to toxic) anesthetic concentrations and because, at toxic concentrations, general anesthetics desensitize the receptor, we focused our attention on the actions of clinically relevant concentrations of general anesthetics on nAcChoR desensitization kinetics.

The Actions of General Anesthetics on the Second Component of Fluorescence

The second component contributes only ~10% of the total fluorescence increase observed between mixing of receptors with Dns-C_6-Cho and the equilibrium achieved approximately 1 min later. The significance of this component has not been defined, but it represents either a binding step (perhaps to preexisting fast desensitized receptors) or a relatively fast conformational transition. Equilibration of receptors with 0.6 mM isoflurane increases the amplitude of this component to 15% (fig. 7). We detected no significant change in the amplitude of this component after equilibration with up to 36 mM butanol.

The rate of this component increases slightly when nAcChoR membranes are exposed to either isoflurane or butanol (fig. 8). A plot of relative rate *versus* anesthetic concentration for either isoflurane or butanol is linear. The relative increase in rate per anesthetic EC_{50} is 18 ± 1.7% and 17 ± 7.8%, respectively, for isoflurane and butanol.

The Actions of General Anesthetics on the Third and Fourth Components of Fluorescence

The amplitudes and rates of the third and fourth components, corresponding to the processes of fast and slow desensitization, respectively, are altered by concentrations of isoflurane and butanol that are in the range required to induce general anesthesia.

Isoflurane and butanol decrease the fractional amplitude of the fourth component over the anesthetic concentration range associated with general anesthesia (fig. 7). For butanol, this decrease is associated with a reciprocal increase in the amplitude of the third component; there is no change in the amplitudes of the first or second components over this concentration range. In the case of isoflurane, the anesthetic actions are more complex, because the amplitudes of the first, second, and third components all increase to varying degrees. The result is that, by 12 mM butanol and 0.6 mM isoflurane, the fractional amplitudes of the third and fourth components become equal in magnitude. Within the framework of model 1, this indicates that general anesthetics increase desensitization *via* fast desensitization kinetic pathways at the expense of the slow desensitization ones. At higher anesthetic concentrations, the third component is greater than the fourth component. At the toxic anesthetic concentrations that greatly increase the fractional amplitude of the first component, the amplitudes of the third and fourth components both diminish to approximately 0.05, a value that is at the limit of our resolution. Only at these toxic anesthetic concentrations could we reasonably fit fluorescent traces to just three components.

For both isoflurane and butanol, the rates of these components increase linearly with increasing general anesthetic concentration (fig. 8). Equilibration of receptors with 0.6 mM isoflurane increases the rates of the third and fourth components by 70 ± 11% and 100 ± 4%, respectively. Similarly, 24 mM butanol increases the rates of the third and fourth components by 70 ± 17% and 130 ± 12%, respectively.

The Actions of Other General Anesthetics of nAcChoR Desensitization Kinetics

Although it was not our goal to survey a large number of general anesthetics, we elected to examine several whose anesthetic potencies varied over a wide concentration range. At twice the EC_{50} for anesthesia in tadpoles, the enhancement in the apparent rates of agonist-induced desensitization is similar for methanol, butanol, chloroform, isoflurane, and cyclopentanemethanol; signal-averaged fluorescence traces nearly superimpose over the entire time course (fig. 9A). For comparison, figure 9B shows the effect of various concentrations of butanol on fluorescent traces. The actions of these anesthetics on the four components of fluorescence are presented in table 2. The increases in the apparent rates of fast and slow desensitization induced by equianesthetic concentrations of these anesthetics range from 70% to 130%. Because these anesthetics differ in their anesthetic potency by approximately 2,000-fold, this represents an impressive correlation with general anesthetic potency. In addition, these anesthetics decrease the fractional amplitude of the fourth fluorescent component and increase the fractional amplitude of the third component. As with isoflurane and butanol, the change in the amplitude and rate of the

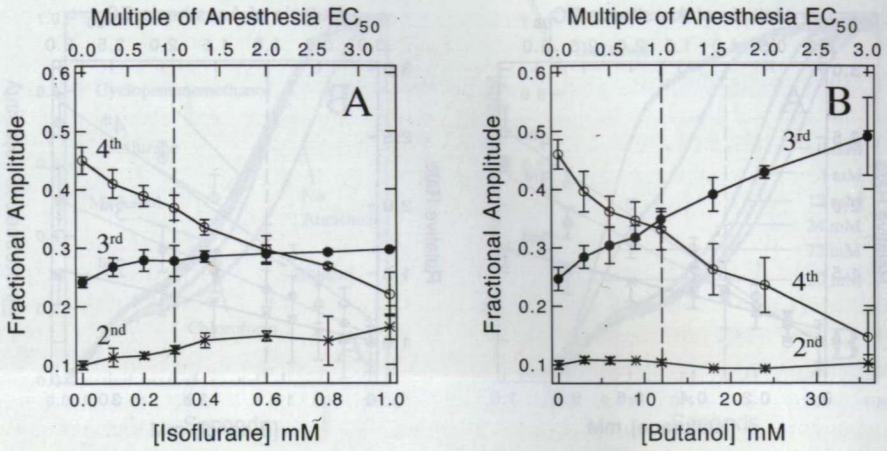

Fig. 7. Change in the fractional amplitude of the second, third, and fourth components after equilibration of receptor membranes with clinically relevant concentrations of isoflurane (*A*) or butanol (*B*). The dashed lines indicate the EC_{50}s for anesthesia in tadpoles, which are 0.3 mM and 12 mM for isoflurane and butanol, respectively. Final concentrations were 2.0 μM Dns-C_6-Cho and 0.4 μM agonist binding sites.

first component after equilibration with either methanol, chloroform, or cyclopentanemethanol at concentrations equal to twice their EC_{50} for anesthesia is not large.

Discussion

Using stopped-flow spectrofluorimetry, we have characterized the actions of general anesthetics on the fraction of nAcChoRs preexisting in the slow desensitized state before agonist-induced desensitization and on agonist-induced desensitization. This fluorescence technique permits agonist binding to be followed essentially continuously from about 1 ms after mixing until equilibrium is reached. The resulting increase in fluorescence that is observed upon mixing membranes containing the nAcChoR with Dns-C_6-Cho reflects agonist binding to desensitized receptors. The time-dependent increase in fluorescence can be adequately described by the sum of four exponentials having time constants ranging from 3 ms to 8 s. The first component occurs on the time scale expected for the diffusion-limited binding of agonist to the receptor. The second component was not resolved in previous studies. Its amplitude is relatively small, contributing only 10% of the fluorescence intensity observed between mixing and equilibrium. Because the K_d for the fast desensitized state is 1 μM which is one-half the concentration of Dns-C_6-Cho used in this study, this component could reflect binding to receptors preexisting in this state.[11,13] The allosteric model depicted in model 1 requires a finite fraction of receptor to be in this state. Previous studies by the Changeux group did not resolve this component.[10,11,13] However, the computers used in their most detailed study did not allow them to signal-average individual shots. It addition, rather than directly analyzing 2,000 points as we did, they digitized fluorescent traces that had been plotted with an x-y recorder and then only fit 20–30 points per trace.[10] These technical limitations would have made detecting this small component difficult. Alternatively, this component may reflect real differences between species; Heidmann and Changeux studied *Torpedo mamorata,* whereas we used *T. nobiliana.*

The third component reflects receptor inactivation occurring on the time scale previously observed with radiotracer flux and single-channel recording techniques.[9,13] This reflects a conformational conversion of resting- and/or open-state channels to the intermediate-affinity, fast desensitized state.[11,13] The fourth component corresponds to the process of slow desensitization observed with relatively low time resolution radioligand assays.

General anesthetics at clinically relevant concentrations significantly alter the kinetics of fast and slow desensitization as reflected in alterations in the third and fourth components of fluorescence. There is a remarkably good correlation between an anesthetic's potency for inducing general anesthesia and that for altering desensitization kinetics. At twice the EC_{50} for general anesthesia, methanol, butanol, isoflurane, chloroform, and cyclopentanemethanol increase the apparent rates of slow desensitization by 92 ± 22% and those of fast desensitization by 108 ± 22%.

Although the processes of agonist-induced fast and slow desensitization are sensitive to clinically relevant concentrations of anesthetics, the fraction of receptors that are in the slow desensitized state before agonist-

for general anesthetic actions on $GABA_A$ receptor currents.[27–30] For example, exposure of $GABA_A$ receptors to 0.96 mM isoflurane enhances the current induced by low concentrations of GABA by 3.5-fold and increases the rate of channel inactivation (desensitization) at high GABA concentrations by 2.6-fold.[31] Normal alcohols have similar actions on $GABA_A$ receptor currents.[32]

The considerable sensitivity of nAcChoR desensitization kinetics to relatively low concentrations of general anesthetics may have important clinical implications. Desensitization of the nAcChoR is believed to play a role in succinylcholine-produced phase II neuromuscular block.[33] Isoflurane and other inhalation anesthetics potentiate succinylcholine-produced phase II block at the same anesthetic concentrations that we demonstrate increase the apparent rates of agonist-induced desensitization of the nAcChoR.[34–36] The latter action may be due to an increase in agonist affinity.[23] If so, then isoflurane may enhance phase II block in part because it increases succinylcholine binding to the receptor. However, the phenomenon of succinylcholine-produced phase II block has not been well characterized on the receptor level, and therefore, any discussion of the relationship between anesthetic-induced enhancement of desensitization and phase II block must be speculative.

The role of desensitization in normal neuromuscular transmission has not been defined, but it is probably not large, because there are excess nAcChoRs at the neuromuscular junction (spare receptors). However, nAcChoR desensitization is a useful model for studying the actions of general anesthetics on the conformational states of ligand-gated ion channels.

In conclusion, general anesthetics increase the apparent rates of fast and slow desensitization. These actions are dose-dependent and occur at clinically relevant general anesthetic concentrations. These results indicate that the nAcChoR, like other members of this superfamily, is a sensitive target of general anesthetics and suggests that the results of a more detailed study of the nAcChoR aimed at understanding the mechanisms underlying the behavior observed here might be of broad significance.

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