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Evidence for Direct Actions of General Anesthetics on an Ion Channel Protein

A New Look at a Unified Mechanism of Action

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Background: **Ion permeation through the nicotinic acetylcholine receptor channel is inhibited by general anesthetics. This inhibition could be mediated either by binding of anesthetic molecules to the channel protein itself or by the effects of anesthetics on the lipid environment of the protein.**

Methods: **Patch clamp recording techniques were used to investigate the effects of ether and propofol on acetylcholine receptor channels in outside-out patches from BC3H-1 cells. The kinetic and conductance properties of single channels were measured. A rapid perfusion system was used to make rapid changes in anesthetic concentration during patch clamp recording to determine the kinetics of inhibition by anesthetics.**

Results: **Ether, isoflurane (results from previous studies), and propofol produce distinct kinetic patterns of single acetylcholine receptor channel activity. Ether reduces the apparent current amplitude of channels, isoflurane induces flickering channel activity and propofol merely decreases the open time of the channel. The kinetics of inhibition are also different for these anesthetics. Ether (<40 μs) is faster than isoflurane (300–600 μs) which is faster than propofol (≥2 ms).**

Conclusions: **These diverse patterns can be interpreted in terms of a unitary mechanism in which the anesthetics interact directly with the channel protein. Each anesthetic is considered to bind to a site on the protein (perhaps, but not necessarily within the pore of the channel) and interrupt the flow of ions through the pore. Anesthetics have access to this inhibitory binding site even when the gate of the channel is closed. The pattern of channel activity induced by an anesthetic is determined by the frequency and duration of binding events. (Key words: general anesthetics, anesthetic mechanisms, ion channels, kinetics.)**

THE question of where general anesthetics exert their effects has been notoriously difficult to answer. The strong correlation between anesthetic potency and oil solubility suggests that the site of action is either hydrophobic[1] or amphipathic[2,3] but provides no further clues about the identity of the site(s). Even if the relevant signaling proteins in the central nervous system were known, it would still be necessary to determine whether general anesthetics act directly on these proteins or indirectly through the lipid membrane surrounding the proteins.[4,5] Perhaps the clearest evidence supporting direct interactions between anesthetics and membrane proteins is based on observations that optical isomers of pentobarbital[6] and isoflurane[7] exert stereospecific actions on ion channel proteins, but not on lipid bilayers. If, indeed, anesthesia is caused by direct actions on proteins, one outstanding issue is whether general anesthetics have a common, unitary mechanism of action.[8]

The nicotinic acetylcholine (ACh) receptor channel is sensitive to general anesthetics and is often used as a model system for studying the molecular mechanisms of anesthetic action.[9,10] The earliest studies examined the effects of anesthetics on end-plate potentials and end-plate currents in muscle cells. The patch clamp technique[11,12] provides a higher resolution method for studying ion channels. Using the patch clamp, it has been shown that a number of general anesthetics induce a flickering pattern of channel activity in ACh receptor channels.[13–16]

Many of the effects of the volatile general anesthetic isoflurane[17] and several *n*-alcohols[15] on ACh receptor channels can be understood in terms of a simple model in which anesthetics bind directly to the ion channel protein. In this model, anesthetic molecules can bind

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to the protein while the gate of the channel is either open (conducting conformation) or closed (nonconducting conformation). When the anesthetic is bound, the gate of the channel continues to operate, but the flow of ions through the channel is blocked. The block might arise from a direct obstruction of the pore of channel by the anesthetic. Alternatively, the anesthetic may bind elsewhere on the channel protein and induce a conformational change to a new, nonconducting state. The channel blocking model also assumes that the gate of the channel closes more readily when anesthetic is bound to the protein.

The open- and closed-channel blocking model interprets flickering as arising from the repeated binding and dissociation of anesthetic molecules to the channel while the channel gate is open. The burst of flickering activity stops when the gate finally closes, most likely while the anesthetic is still bound. This would account for the short duration of bursts in the presence of anesthetic—an observation that cannot be explained by a pure open-channel blocking model.[14,15,17]

Here, we test whether the open- and closed-channel blocking model can be used to describe the actions of other general anesthetics. We have chosen two anesthetics: ether, which is less potent than isoflurane, and propofol, which is more potent than isoflurane. We examine the actions of these anesthetics on single ACh receptor channels and also on the macroscopic currents that flow through an ensemble of open channels. The latter approach allows direct measurement of the rates of anesthetic binding and dissociation and determination of whether these rates are consistent with the open- and closed-channel blocking model.

Materials and Methods

Clonal BC3H-1 cells expressing nicotinic ACh receptor channels were prepared for patch clamp recording.[17] Culture medium was replaced with an "extracellular solution" containing (millimolar) NaCl (150), KCl (5.6), $CaCl_2$ (1.8), $MgCl_2$ (1.0) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (10), *p*H 7.3. Patch pipettes were filled with a solution consisting of KCl (140), EGTA (5), $MgCl_2$ (5) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (10), *p*H 7.3 and had resistances of 4–6 MΩ. An outside-out patch[18] was excised from the cell and brought into position at the outflow tube of a perfusion system. For the single-channel experiments, the perfusion system consisted of reservoirs containing 200 nM ACh and the desired concentration of anesthetic in extracellular solution, a manual switching valve and plastic tubing.[17] A concentration of 200 nM ACh is low enough to produce recordings consisting of mostly isolated single-channel openings. For each patch, control channel activity at −100 mV applied potential was recorded before and after exposure to anesthetic. Recovery from drug inhibition was considered complete when the channel kinetics for the two control data sets differed by no more than 20%. Single-channel currents were amplified, filtered at 3 kHz (−3 dB, eight-pole Bessel filter), digitized at 50 μs per point and stored on a computer hard disk. Data analysis was performed offline as described elsewhere.[17] The resolution for channel opening events was 100 μs, the resolution for channel closing events was 50 μs. Channel duration histograms are displayed using a log-binned time axis.[19] Amplitude histograms for ether were analyzed using a procedure to estimate the blocking and unblocking rates of fast channel blockers.[20]

A system for rapid perfusion of outside-out patches[21,22] was used to examine the kinetics of anesthetic inhibition of channels. In this system, a solenoid-driven pinch valve is used to change the solution bathing the patch from normal (extracellular solution alone) to test solution (extracellular solution plus 10 mM ACh). In the experiments described here, the solution exchange is complete within 100 μs.[22] The currents measured during rapid application of 10 mM ACh (a concentration high enough to saturate the ACh-binding sites on the receptor) arise from the activation of nearly all of the 50–100 channels in the patch. We refer to such currents as "macroscopic" currents because single-channel events cannot be discerned. Anesthetics were included in either the normal or test solution (or both) as indicated. Macroscopic currents at −50 mV applied potential were amplified, filtered at 10 kHz (−3 db, eight-pole Bessel filter), digitized at 5 μs per point and stored on a computer hard disk. The protocol was to obtain five ensembles of current records with the following combinations of normal and test solutions (where − = solution without anesthetic and + = solution with anesthetic): first control (normal−, test−), equilibrium (normal+, test+), onset (normal−, test+), recovery (normal+, test−), and second control (normal−, test−). The order of the equilibrium, onset and recovery runs was varied. An experiment was considered successful when the amplitudes of the first and second controls differed by no more than 20%. For each condition, we recorded an

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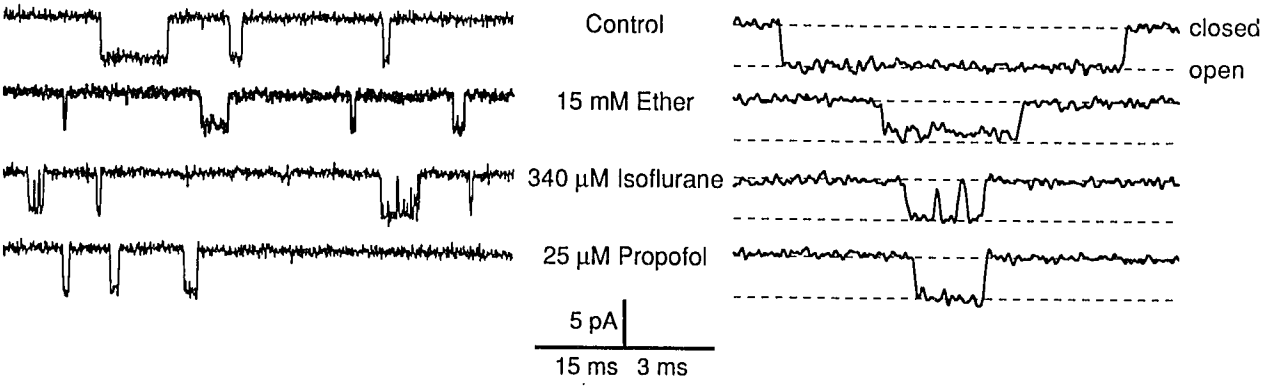

Fig. 1. Single ACh receptor channels recorded from outside-out patches from BC3H-1 cells, activated by 200 nM acetylcholine and perfused with no drug (control), 15 mM ether, 340 μM (0.7%) isoflurane (data from reference 17), or 25 μM propofol. The control and propofol records come from the same patch. Channel openings are shown as downward transitions. (*Right*) Traces show one of the bursts (from *left*) on an expanded time scale. Dashed lines = current amplitude in the control record, −4.2 pA.

ensemble of 15 records in which ACh was applied to the patch for 40 ms. The records were obtained 1 s apart.

Technical concerns dictate the voltages used in the experiments. Single-channel measurements are performed at −100 mV to achieve a signal-to-noise ratio high enough for single-channel analysis. There is very little voltage-dependence (between −100 and +100 mV) for the effects of isoflurane on single channels.[17] Macroscopic current measurements are performed at −50 mV to minimize the blocking effects of 10 mM ACh.[22] However, macroscopic currents are always acquired at both −50 mV and +50 mV (there is less than 10% block by 10 mM ACh at +50 mV). The currents recorded at +50 mV are not presented here but are similar to those recorded at −50 mV, scaled by a factor of −2 to account for block by ACh and the opposite voltage polarity.[23]

Experiments were performed at room temperature (20–24°C). Aliquots of ether (diethyl ether; reagent grade, Fisher Scientific, Fair Lawn, NJ) and propofol (2,6-diisopropylphenol; purity 97%, containing no solvent; Aldrich Chemical, Milwaukee, WI) were added directly to aqueous solutions. Reservoirs containing ether were sealed to prevent evaporation. Solutions containing propofol were stirred vigorously overnight in a closed glass bottle to insure that the propofol was completely dissolved. This procedure produced a solution that did not scatter light. The results of experiments using isoflurane are taken from previously published results from this laboratory obtained and analyzed using the same methodologies.[17,23]

The potency of these drugs for inducing general anesthesia (expressed as aqueous concentrations) are: 9.3 mM ether[24] (human MAC), 0.28 mM isoflurane[24] (human MAC) and 0.0022 mM propofol[25] (tadpole EC_{50}).

Results are expressed as means ± standard deviations with the number of measurements given in parentheses.

Results

Figure 1 shows examples of single nicotinic ACh receptor channels under control conditions and during exposure to 15 mM ether, 340 μM isoflurane (0.7%) or 25 μM propofol. Normally, channels stay open for several milliseconds and are infrequently interrupted by brief gaps. Previously, we found that in the presence of isoflurane, channel activity occurs in bursts, a series of short openings of a single channel separated by somewhat longer than normal gaps.[14,17] Isoflurane makes the channels "flicker," but has little effect on the current amplitude of the channel. In the presence of the less potent general anesthetic, ether, the channels appear to have a smaller, noisier amplitude than in control. With the highly potent general anesthetic propofol, channels appear as isolated brief openings, with no evidence of either flickering or decreased amplitude.[16]

Figure 2 shows the anesthetic concentration dependence of the mean open duration, the number of openings per burst, and the single-channel current amplitude. These data show that the kinetic pattern induced by an anesthetic is not due to the concentration used.

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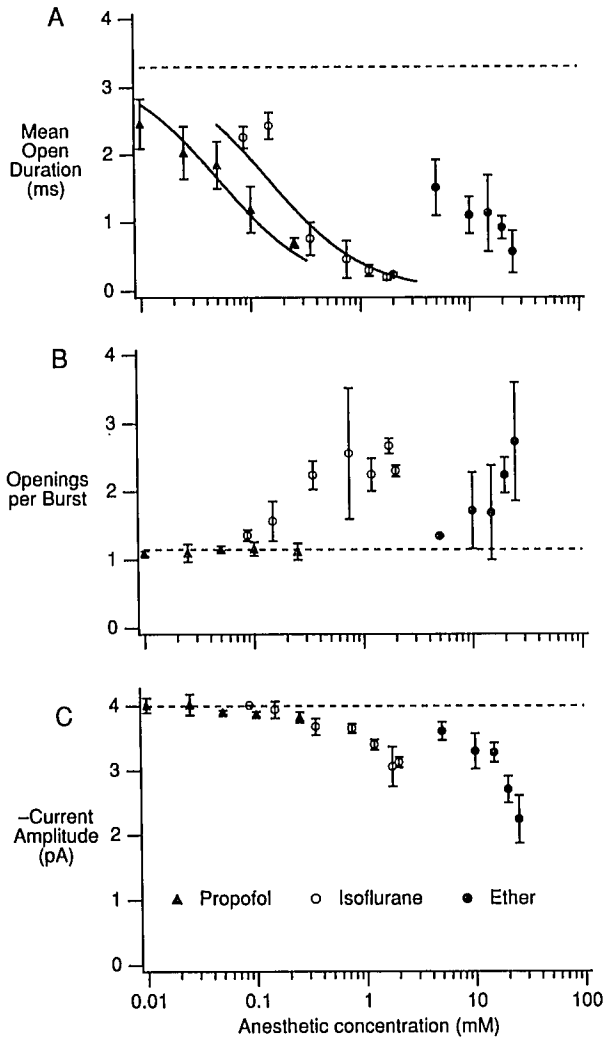

Fig. 2. The concentration dependences of some properties of ACh receptor channels exposed to ether, isoflurane (data from reference 17), or propofol. Data are presented as means ± SD for three to eight patches at each anesthetic concentration. Dashed lines = measured control values. (*A*) Mean open duration. Solid lines for propofol and isoflurane = fits of the data to scheme 1 with the association rate constants of 6×10^6 M$^{-1}\cdot$s^{-1} and 2×10^6 M$^{-1}\cdot$s^{-1}, respectively. The data for ether are not fit to scheme 1 because most of the blocking events by ether are not resolved. Thus, the mean open durations for ether are overestimates. (*B*) Number of openings per burst. (*C*) Single-channel current amplitude (absolute value).

Neither lower concentrations of ether nor higher concentrations of propofol produces the sort of flickering induced by isoflurane.[17] In particular, 250 μM propofol reduces open time to the same extent as 340 μM isoflurane but there is a dramatic difference between the number of openings per burst (amount of flickering) induced (1.1 ± 0.1 for 250 μM propofol and 2.2 ± 0.2 for 340 μM isoflurane). Also, we observe some flickering with 5 mM ether (1.3 ± 0.02 openings per burst), but not as much as that seen with even lower concentrations of isoflurane (*e.g.*, 2.7 ± 0.1 openings per burst at 1.8 mM isoflurane). Higher concentrations of ether induce flickering (as many as 2.7 ± 0.9 openings per burst at 25 mM ether), but this is accompanied by a large decrease in current amplitude (to −2.2 ± 0.4 pA).

Channel flickering can be studied quantitatively by constructing histograms of closed-time durations for a long recording of single-channel activity. Examples of these histograms are displayed in figure 3. The number of peaks (components) in a closed-time histogram indicates the minimum number of different nonconducting states of the channel. The closed time histogram of control recordings has two components; the larger component at 100 ms corresponds to the time between independent channel openings, the smaller component at 0.1 ms corresponds to the brief gaps seen in 5–10% of the control bursts. The duration of the long component varies from patch to patch depending on the number of channels in the patch and is not considered further here. The brief component contains information about repeated openings of a single channel within a burst. Under control conditions, the average gap duration is similar to the time resolution of the recording system (50 μs) so some gaps are too brief to be detected.

The closed time histogram (fig. 3) compiled from recordings of the same patch exposed to 15 mM ether reveal an increased frequency of brief gaps. The average duration of these gaps is estimated to be only 20 μs, so, the brief gap component is not completely resolved. In the closed time histogram for 340 μM isoflurane,[17] brief gaps are more numerous and they have an average duration of 200 μs. There is only a small brief gap component in the closed-time histogram for 25 μM propofol; it is similar to the one seen in the control histogram.

The appearance of channels exposed to ether, (a smaller-than-normal, noisy amplitude) is characteristic of the effect of a drug that induces very rapid channel flickering.[20] For such a drug, it is usually assumed that a burst consists of many full-amplitude openings and many complete interruptions of the current. However, the openings and interruptions are so brief that most of them are attenuated by the low-pass filter (set at 3 kHz in our experiments) or not detected (the digitizing time is 50 μs in our experiments). The result is that

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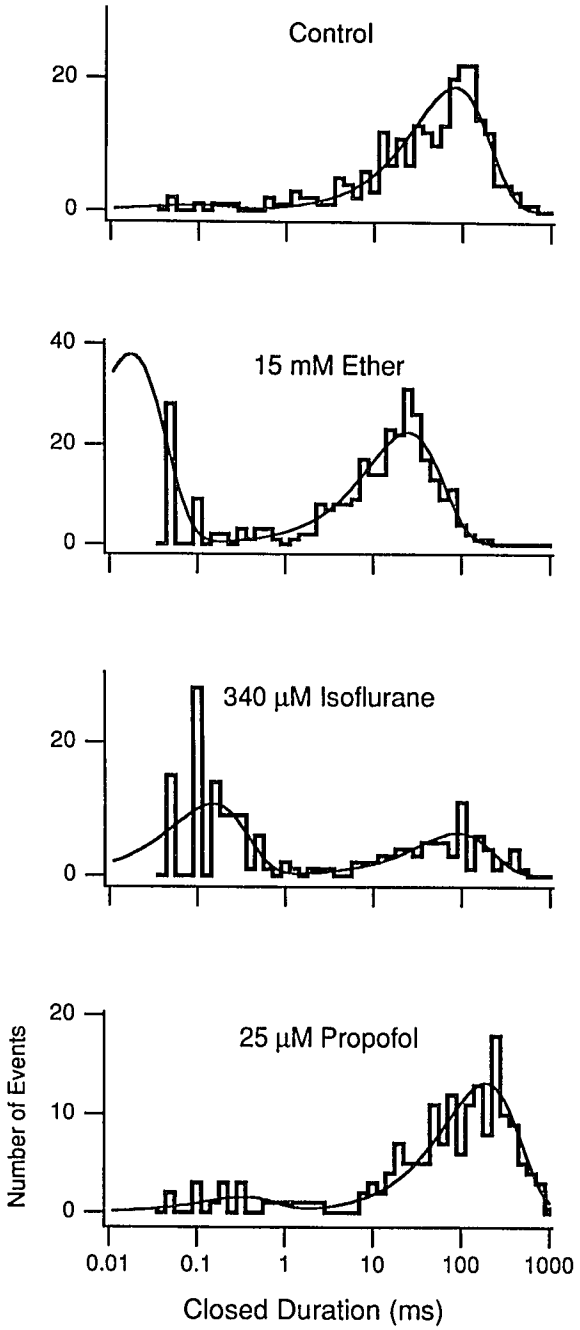

Fig. 3. Closed duration histograms of channel activity in control and in the presence of 15 mM ether, 340 μM (0.7%) isoflurane (data from reference 17), or 25 μM propofol. When plotted on a logarithmic time scale, the double-exponential distributions of closed times appear as a pair of peaks. The time at the maximum height of each peak corresponds to the time constant or mean duration of one of the closed time components. Smooth lines = double-exponential fits to the distributions corrected for unresolved closures.[19] The control and propofol histograms are from the same patch.

the channel *appears* to have a reduced amplitude. Experiments using a higher frequency resolution can, in principle, determine the validity of this interpretation. However, the high level of background noise in such experiments makes the analysis of channel events more difficult. An alternate approach to quantifying the effects of putative fast blockers is to analyze the current amplitude distribution of the data (fig. 4). These histograms consist of two peaks, one centered at 0 pA corresponding to the baseline current when no channels are open (this is only partially seen in the figure) and one centered at a negative current amplitude corresponding to the current level when just one channel is open. In control recordings, the single-channel amplitude distribution can be described by a Gaussian (normal) distribution with a mean of −4.1 pA and a standard deviation of 0.7 pA. In the presence of 25 mM ether, the mean amplitude is smaller, −2.6 pA, but the standard deviation is larger, 1.1 pA. The additional width of this distribution corresponds to the higher level of noise seen in single-channel records. If the

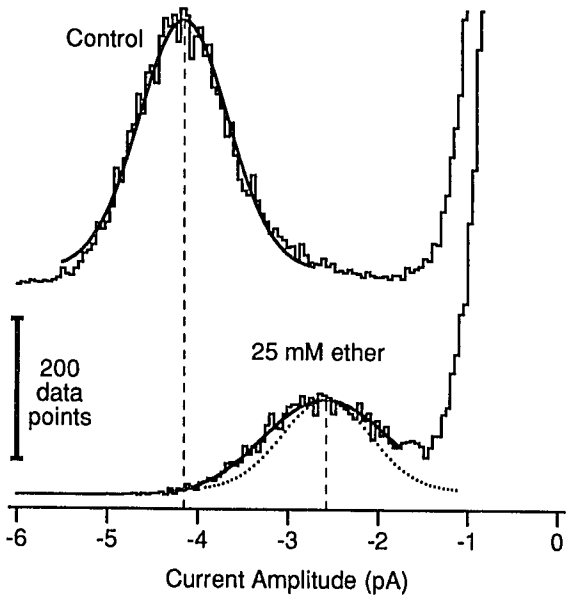

Fig. 4. Amplitude histograms obtained from a patch exposed to 0 and 25 mM ether. Bins are 0.05 pA wide and contain sampled data points from single-channel recordings filtered at 3 kHz. Peaks at 0 pA, which correspond to the baseline currents, are not completely displayed. The control histogram is fit to a Gaussian distribution; the 25 mM ether histogram is fit to a β function (solid curves). Dotted curve = control histogram shifted by 1.6 pA along the amplitude axis and scaled by a factor of 0.36 on the y-axis. We interpret the broader width of the ether histogram as resulting from partially resolved blocking and unblocking events.

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noise arises from incompletely resolved fast flickering between open and closed states, then the amplitude distribution can be fit to another type of distribution.§ This distribution is determined by two parameters: the blocking and unblocking rates of the drug. In fitting the data of figure 4, we found a blocking rate of 70 ms^{-1} and an unblocking rate of 110 ms^{-1}. In 11 patches exposed to 15–25 mM ether, the mean association rate (blocking rate divided by ether concentration) was (2.3 ± 0.5) × 10^6 $M^{-1} \cdot s^{-1}$, and the mean unblocking rate was (1.0 ± 0.2) × $10^5 \cdot s^{-1}$.

The rates of onset and recovery of anesthetic action were determined using a method for making rapid changes of solution surrounding an excised membrane patch during patch clamp recording. Under ideal conditions, complete solution exchange can be achieved in less than 100 μs.[22] Figure 5 shows some examples of macroscopic currents under control conditions (the two larger amplitude traces in each panel). In these experiments, we use a supersaturating concentration of agonist (10 mM ACh) to activate nearly all of the channels in the patch within 40 μs.‖ The slight decrease of current seen at the end of each trace results from a desensitization process that occurs on the 50–100-ms time scale.[26] The two control traces show that the currents were restored to ≥80% of their original amplitude after removal of anesthetic.

The smallest amplitude trace in each panel of figure 5 shows the macroscopic currents under conditions where the patch is constantly exposed to 20 mM ether, 1 mM isoflurane,[23] or 100 μM propofol. These currents represent the response to ACh during equilibrium exposure to anesthetic. In the case of ether and isoflurane, the macroscopic current is reduced by about 40% from the first control throughout the 5 ms after agonist application. The degree of current suppression by propofol changes from 50% at the beginning of the trace to 70% at the end of the trace (compared with the first control). The control (average of the two controls) and equilibrium traces serve as reference levels for the degree of current suppression by the anesthetics. These traces are shown in figures 6 and 7.

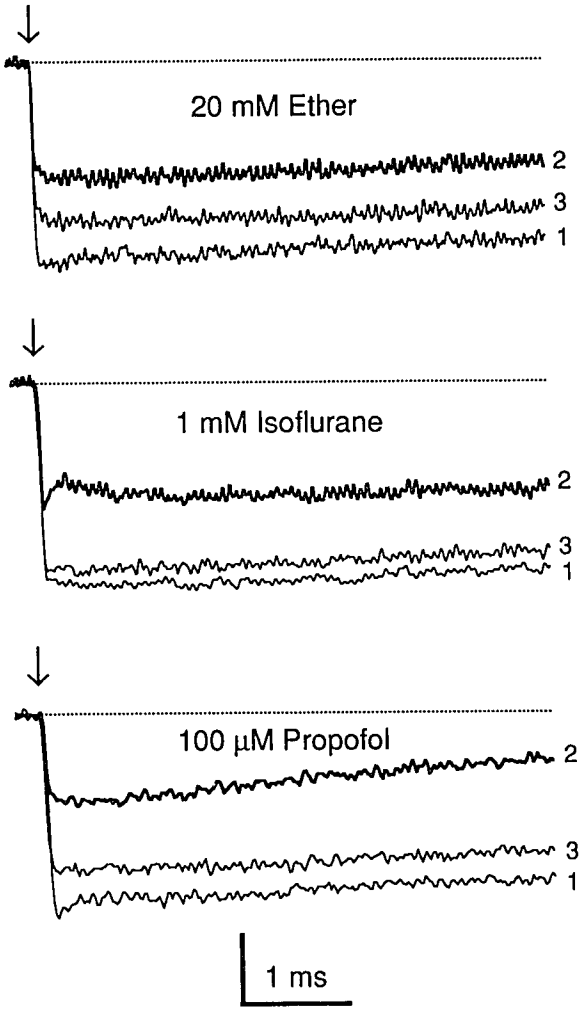

Fig. 5. Equilibrium inhibition of macroscopic currents by general anesthetics. At the time indicated (arrow), 10 mM ACh was perfused onto patches containing about 80 ACh receptor channels. The two larger amplitude traces in each panel were obtained before (*1*) and after (*3*) applications of anesthetic. The smaller amplitude traces (*2*) were obtained during constant exposure of the patch to the indicated concentration of anesthetic. Dotted lines = baseline current level. Data in each panel come from three patches, one for each anesthetic. Data from these three patches also are shown in figures 6 and 7. The data for isoflurane are from reference 23. Vertical calibration bar = 28, 27, and 21 pA for ether, isoflurane, and propofol, respectively.

Figure 6 shows the effects of rapidly applying anesthetics to the patch to monitor the onset of current suppression. The experimental protocol was to perfuse the patch with anesthetic-free solution before ACh was

§ The amplitude distribution is fit to a β distribution, convoluted with a Gaussian distribution with a standard deviation equal to the standard deviation of the baseline noise.[20] The calculated rates depend on the filter frequency.

‖ Although it may take 100 μs to complete the solution exchange, the channels see a saturating concentration of ACh (500 μM) much sooner than that and the current risetime is limited by the rate at which channels open, 40 μs.

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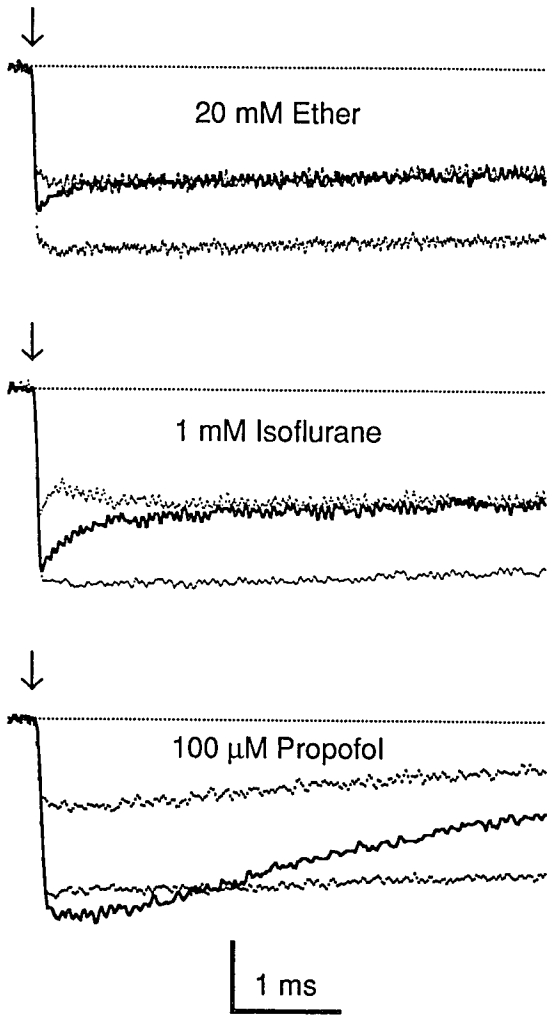

Fig. 6. Onset of inhibition of macroscopic currents by general anesthetics. Before perfusion of acetylcholine, the patch was perfused with anesthetic-free solution. At the time indicated (arrow), the patch was perfused with 10 mM acetylcholine and the indicated concentration of anesthetic. Light traces = control (average of two controls) and equilibrium currents from figure 5. The peak amplitude of the propofol onset trace is the same level as the amplitude of the first control trace in figure 5. The data for isoflurane are from reference 23. Vertical calibration bar = 28, 27, and 21 pA for ether, isoflurane, and propofol, respectively.

applied and to include the specified concentration of anesthetic in the ACh perfusion solution. The time course of the onset current trace indicates the speed with which the anesthetic reaches its site of action and suppresses the current. For ether, the onset current trace is almost indistinguishable from the equilibrium trace. Thus, ether equilibrates rapidly with its site of action; equilibration is 75% complete within 50 μs. For isoflurane, the onset current decays from the control level to the equilibrium level with a time constant of 300 μs.[23] With propofol, the onset current decays from the control level to the equilibrium level much more slowly; the decay time constant is about 4 ms. A longer current trace (not shown) indicates that the equilibrium current level is reached after about 15 ms.

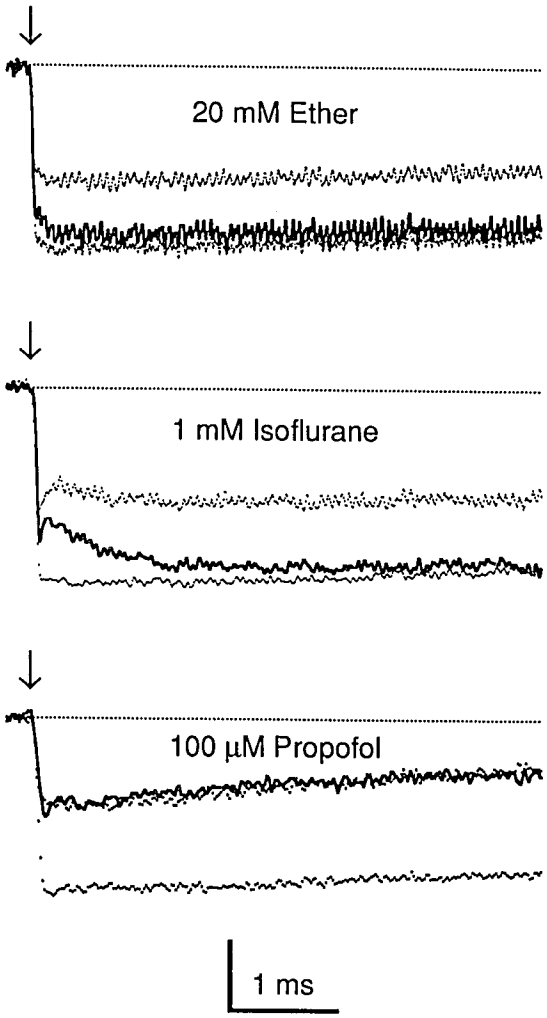

Fig. 7. Recovery from inhibition of macroscopic currents by general anesthetics. Before perfusion of acetylcholine, the patch was perfused with a solution containing the indicated amount of anesthetic. At the time indicated (arrow), the patch was perfused with a solution containing 10 mM ACh and no anesthetic. Light traces = control (average of two controls) and equilibrium currents from figure 5. The data for isoflurane are from reference 23. Vertical calibration bar = 28, 27, and 21 pA for ether, isoflurane, and propofol, respectively.

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Figure 7 contains macroscopic current traces obtained during rapid removal of anesthetic from the patch. In these experiments, the patch was perfused with the specified concentration of anesthetic before ACh was applied but the ACh perfusion solution did not contain anesthetic. The time course of the recovery current trace indicates the speed with which the anesthetic leaves its site of action. The current obtained after removal of ether quickly recovers to within 95% of the control current. When isoflurane is rapidly removed, the current relaxes from the equilibrium level to the control level with a time constant of 750 μs.[23] In contrast, there is no recovery from inhibition by propofol within 5 ms after removal of propofol. A longer trace (not shown) reveals partial recovery of the current over 40 ms but the complete time course of recovery of inhibition by propofol is obscured by the fast desensitization of ACh receptor channels. Macroscopic currents measured in the presence of 10–75 μM propofol show less inhibition of current than 100 μM propofol, but recovery from inhibition is also on the ≥10 ms time scale.

Inhibition by propofol is reversible. The macroscopic current returns to the control level at some point within the 5 min that elapsed between measurements of the equilibrium and second control current traces (fig. 5). In addition, in the onset experiments (fig. 6), the effects of single 20-ms exposures to propofol were reversed before each subsequent 20-ms exposure was made 1 s later.

We examined the kinetics of inhibition by 20 mM ether, and 100 μM propofol in 4–13 patches with each anesthetic. The results were qualitatively similar to those shown in figures 5–7: the kinetics of ether were too fast to be measured and the kinetics of propofol were too slow to be measured with our rapid perfusion protocol. With 1 mM isoflurane, we previously found an average onset time constant of 200 ± 100 μs (n = 10) and recovery time constant of 480 ± 160 μs (n = 13).[23]

Discussion

Ether, isoflurane, and propofol produce distinct effects on single ACh receptor channels (fig. 1). At first, this might be considered evidence that these anesthetics affect the channel in very different ways—*i.e.*, that the mechanisms of action of these anesthetics differ. We use our results to support the idea that a single mechanism *can* be used to explain the actions of all three anesthetics and that the observed differences are a consequence of the rates at which the anesthetics bind to and dissociate from a common binding site.

Scheme 1 (fig. 8) depicts the open- and closed-channel blocking model that has been used to describe the action of isoflurane[17] and *n*-alcohols from pentanol through dodecanol[15] on ACh receptor channels. The top limb of this scheme describes the normal function of the ACh receptor channel.[27–30] Agonist-binding and channel-gating are two separate processes. Two agonist molecules bind sequentially to binding sites on the receptors to form a protein state that can isomerize to an open-channel conformation. The states in the bottom limb of the scheme represent each of the four normal channel states with one anesthetic molecule bound. The anesthetic may bind with different affinities to different states but it is assumed that none of the anesthetic-bound states can conduct current. The anesthetic may either physically obstruct the pore of the channel or bind elsewhere on the protein to induce a new, closed conformation of the channel. When the anesthetic molecule is bound, channel-gating and agonist-binding may occur with somewhat different rates than normal.

At low concentrations of ACh, channel activation is infrequent because it requires the binding of two agonist molecules within about 25 μs. Once binding occurs, there is about a 40% probability that the gate of the channel will open and remain open for a few milliseconds. After the gate closes, the most probable event is for the agonist molecules to dissociate (one at a time, in quick succession), but occasionally, the gate of the channel will reopen within about 50 μs. This reopening gives rise to the brief gaps sometimes seen in control channel recordings.

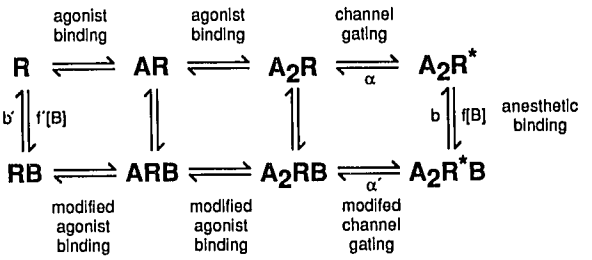

Fig. 8. Scheme 1. A = agonist (acetylcholine); R = receptor; * = open-channel conformation; B = anesthetic molecule; α = normal channel-closing rate; α' = closing rate of the anesthetic-bound channel; f and f' = rate constants for the binding of anesthetic to the open or the closed channel, respectively; b and b' = rate constants for anesthetic dissociation from the open or the closed channel, respectively.

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Scheme 1 describes flickering by an anesthetic such as isoflurane as follows. While the gate of the channel is open (state A_2R^*), isoflurane molecules repeatedly bind (which interrupts current flow) and dissociate (which restores current flow) giving rise to a burst of channel openings. The duration of the gaps within the burst depends on the length of time isoflurane remains bound. Certain aspects of channel kinetics indicate that the gate of the channel closes more readily when isoflurane is bound ($\alpha' > \alpha$).[17] Thus, it is more likely that a burst ends when agonist molecules dissociate from isoflurane-bound channel ($A_2R^* \rightarrow A_2R^*B \rightarrow A_2RB \rightarrow$ ARB or AR).

Scheme 1 can also account for the patterns of channel activity seen with ether and propofol. Suppose that 15 mM ether binds more frequently and for shorter periods of time than 340 μM isoflurane. The gaps within a burst would be numerous, but very brief. If most of these gaps were shorter than the time resolution of the current recording system, then individual binding and dissociation events would usually not be detected. Although each binding event completely stops current flow through the channel, channels would *appear* to have a noisy, smaller than normal amplitude. Suppose that propofol remains bound for a longer duration than isoflurane and even longer than the normal open time of the channel. Channel openings would be briefer than normal, but flickering would not be seen. For both ether and propofol, the burst would end when the drug-bound channel closes and the ACh dissociates. In terms of scheme 1, ether, isoflurane, and propofol would be characterized as fast, intermediate and slow blockers respectively.

Scheme 1 predicts that the mean channel open time (τ_o) is given by $\tau_o = (\alpha + f[B])^{-1}$, where B = anesthetic concentration. The association rate constant for the anesthetic (f) can be determined from the concentration dependence of τ_o. For propofol and isoflurane, f is obtained by fitting the data in figure 2. For a fast blocker like ether, the measured open duration is an overestimate of the actual open duration because many of the brief openings and closures are not detected. For ether, we use the results of fitting the current amplitude histogram to estimate the association rate constant. The three anesthetics have very similar association rates as might be expected for the diffusion of molecules of similar size (table 1).

Scheme 1 predicts that, at high concentrations of ACh, the equilibrium level of current inhibition depends on both the association and dissociation rate constants:

Table 1. The Association and Dissociation Rate Constants for Inhibition of Acetylchloline Receptor Channels by General Anesthetics

Anesthetic	f (mM^{-1} ms^{-1})	b (ms^{-1})	b/f (mM)	α/f (mM)
Ether	2.3	100	43	0.13
Isoflurane	2.0	2	1	0.16
Propofol	6.0	≤0.6	≤0.1	0.05

f = association rate constant; b = dissociation rate constant; b/f = equilibrium binding constant (50% inhibition concentration for macroscopic currents); α/f = ratio of the channel closing rate, α (310 ms^{-1}), and f. (This ratio gives the 50% inhibition concentration for the mean open time of single channel currents.) Values for isoflurane are from reference 17.

equilibrium inhibition = $(1 + f[B]/b)^{-1}$.[23] The equilibrium currents shown in figure 5 for isoflurane and propofol correspond to about 50% inhibition; we use this to calculate the dissociation rate constant (b). For ether, b is obtained from analysis of the current amplitude histogram. (These values of b and f predict that 43 mM ether would produce 50% inhibition of the macroscopic current). The dissociation rates for ether, isoflurane, and propofol are quite distinct (table 1). Ether dissociates 50 times more quickly than isoflurane, propofol dissociates at least 3 times more slowly than isoflurane. The most hydrophobic of the three drugs, propofol, dissociates at the slowest rate. This dissociation pattern suggests that the chemical nature of the binding site is at least partly hydrophobic.

If scheme 1 is an appropriate model for the action of anesthetics on the ACh receptor channel, then the binding and dissociation rates listed in table 1 should predict the kinetics of onset and recovery of current inhibition upon rapid addition and removal of anesthetic. The onset of current inhibition, $(f[B] + b)^{-1}$, is 7 μs for 20 mM ether, 250 μs for 1 mM isoflurane, and 830 μs for 0.1 mM propofol. Recovery from current inhibition, b^{-1}, is 10 μs for ether, 500 μs for isoflurane, and ≥1,700 μs for propofol. For ether, both onset and recovery kinetics are predicted to be faster than the time resolution of the rapid perfusion apparatus; the data in figures 6 and 7 qualitatively support this. For isoflurane, the predicted kinetics quantitatively agree with our observed values of 200 μs for onset and 480 μs for recovery.[23]

For propofol, however, the observed onset and recovery rates (figs. 6 and 7) are slower than the predicted kinetics. Although this might indicate the inadequacy of scheme 1 to describe the effects of propofol, there are two other factors that may account for

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this discrepancy. First, our estimate of the dissociation rate for propofol is based on the concentration of propofol needed to block 50% of the open channels. The decay in figure 5 indicates that 100 μM propofol blocks 50% of the closed channels, but a higher fraction of the open channels (in contrast, 20 mM ether blocks both open and closed channels by 35% and 1 mM isoflurane[23] blocks both open and closed channels by 40%). Desensitization of the ACh receptor obscures the relaxation from closed- to open-channel block so we cannot determine just how much propofol is required to block open channels. Macroscopic current experiments with 10–50 μM propofol, however, suggest that the equilibrium blocking concentration of propofol on open channels is no lower than 50 μM. This is not low enough to account for the slow kinetics of propofol.

Alternatively, consider the high oil solubility of propofol. The octanol/water partition coefficient of propofol is 4,300[25]; much greater than that of isoflurane (170) or ether (7.8).[24] When 100 μM propofol is present in the aqueous phase, there is 430 mM propofol in the membrane. The slow recovery kinetics for propofol might reflect the time needed for this large reservoir of propofol to be depleted. We have observed a slow recovery rate for inhibition by octanol as well.[31] We do not know of any direct measurements of the equilibration time for propofol between water and the membrane, but based on the upper limit for the equilibration time for halothane of 5 ms,[32] an upper limit of 50 ms for propofol might be reasonable.

The concentrations used in the macroscopic current experiments (figs. 5–7) are higher than and are not simply related to those used in the single-channel experiments (fig. 1). This raises the possibility that the drug effects being studied by the two techniques are different. Although we cannot rule out that possibility, our observations are consistent with scheme 1. Table 1 includes the predictions of scheme 1 for the drug concentration needed to reduce macroscopic currents by 50% (b/f). The final column of table 1 gives the predictions of scheme 1 for the drug concentration needed to reduce open-channel durations by 50%, α/f. These concentrations depend on the closing rate of the channel (α) as well as on the association rate of the drug. Thus, scheme 1 predicts that both 130 μM ether and 50 μM propofol should reduce open time by 50%. This is observed for propofol (fig. 2). However, scheme 1 predicts channels are blocked by ether for an average of only 10 μs—a blocking event that could not be detected in our single-channel recording experiments.

Because the kinetics of ether are so fast, the number of detected openings per burst (fig. 2B) underestimates the actual number. With 10 mM ether, the rate constants derived for ether (table 1) predict that ether binds about 20 times per millisecond. The burst duration in the presence of 10 mM ether is 1.6 ms, so the predicted number of openings per burst is 33. If we could measure single-channel currents with a microsecond time resolution, we would find that the average burst consists of 33 separate openings, each lasting 50 μs and separated by 10 μs gaps.

Scheme 1 has a long history as a postulated mechanism for the action of drugs on ion channel proeins.[33–35] It is often considered after a purely open-channel blocking mechanism (scheme 1 with only the A_2R^*B state in the bottom limb) has been considered and found inadequate. However, there is often little direct evidence for the validity of scheme 1. Here, we show that two essential features of scheme 1 are valid for ether, isoflurane, and propofol. First, the rate of channel flickering corresponds to the rates of drug binding and dissociation. Second, drugs bind to closed as well as open channels. The first feature was discussed above. Figure 5 provides evidence that these anesthetics are capable of binding to closed states of the channel. Because the equilibrium macroscopic currents are suppressed as soon as the agonist is perfused, the anesthetics (at least isoflurane and propofol) must have equilibrated with their binding site before the opening of the gate of the channel.

Other general anesthetics produce the patterns of ACh receptor channel activity seen in figure 1, and the pattern produced appears to be related to the potency of the drug as an inhibitor of the channel. The more potent drugs, fentanyl[16] and long chain alcohols[15,36] produce isolated brief openings similar to propofol. Drugs with intermediate potency, enflurane,[36] halothane[37,#] and hexanol[15,36] produce flickering. The less potent drugs, benzyl alcohol[40] and butanol[36] produce an apparent decrease in channel amplitude like ether.

The action of halothane on ACh receptor channels appears to be different on ACh receptor channels from different preparations. Halothane produces flickering in channels from BC3H-1 cells[37] but only reduces the open time of channels from embryonic *Xenopus* muscle cells[38] and rat myoballs.[39] The origin of this difference is not yet known. The preparations also differ in the concentrations of halothane needed to produce an effect: channels from BC3H-1 cells are more sensitive to halothane than are channels from either *Xenopus* muscle or rat myoballs.

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We have argued that the diversity of the effects of ether, isoflurane, and propofol on ACh receptor channels is consistent with the idea of a unitary mechanism of action for these anesthetics. Such a diversity would not be expected if the anesthetics had a lipid site of action, however. If the anesthetics simply dissolved in the lipid bilayer membrane and perturbed the membrane in some way (say, increased its fluidity or volume to some critical value), the effect of equipotent concentrations of any anesthetic on channel behavior would be the same. It is conceivable that the three anesthetics exert their diverse effects by perturbing the lipid bilayer in three different ways. Such an unconventional extension of a lipid-based hypothesis for the actions of anesthetics would also have to account for our observation that the duration of flickering gaps induced by isoflurane (fig. 3) is the same as the time needed for the channels to recover after removal of isoflurane (fig. 7). A simpler interpretation of the apparent specificity seen with these anesthetics on ACh receptor channels is that the anesthetics are acting either directly on the protein itself or at the interface between the protein and the membrane.

The muscle ACh receptor is clearly not the primary target of general anesthetics. Given that this channel is the prototypical ligand-gated ion channel, though, it is likely that general anesthetics may also have direct effects on other members of the ionotropic channel protein superfamily.[41]

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