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Effects of Isoflurane on Acetylcholine Receptor Channels. 1. Single-Channel Currents

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SUMMARY

We studied the effects of the volatile general anesthetic isoflurane on single acetylcholine (ACh) receptor channels from clonal BC3H-1 cells. Excised patches were exposed to concentrations of isoflurane ranging from 0.18% to 4.0%, in the presence of 200 nM ACh. Isoflurane transformed channel behavior from isolated openings into bursts of brief openings. The channel open time decreased monotonically with the concentration of isoflurane; the mean open time was half of control at 0.4% isoflurane. The duration of bursts also decreased in the presence of isoflurane. The duration of brief closures within bursts was 300–400 μsec at concentrations above 0.3% isoflurane. The number of openings per burst increased moderately with isoflurane but did not exceed 3. The frequency of bursts increased with the concentration of isoflurane. The apparent single-channel conductance decreased to 75% of control at 4% isoflurane. These results are discussed in terms of models of channel block. The concentration dependence of the open time, the gap duration, and the conductance are consistent with a sequential open-channel blocking mechanism in which most but not all blocking events were resolved. A model that assumes that isoflurane "blocks" both open and closed channels was then considered. This model is consistent not only with the open time data but also with the burst duration and number of openings per burst. These results indicate that isoflurane has effects on closed as well as open ACh receptor channels.

Despite decades of study by many investigators, the mechanisms by which general anesthetics produce unconsciousness are unknown. Part of the difficulty is that our knowledge of the neuronal processes underlying consciousness and behavioral states is still scanty. The problem is complicated by the large number and structural diversity of compounds that give rise to these states. The Meyer-Overton Rule (1), which states that the potency of anesthetics is strongly correlated with their lipid solubility, provided a clue, that the "sites" from which anesthetics exert their characteristic actions are hydrophobic. Initially, this was widely interpreted to mean that a single site, the lipid bilayer, might be the target of many anesthetics. More recently, the interpretation has broadened to include hydrophobic regions of proteins as possible sites for anesthetic binding (2).

Because ion channels are among the important signaling molecules in the nervous system, they are likely targets for general anesthetics. The availability of sensitive techniques for assaying ion channel structure and function makes ion channels attractive model systems for quantitative tests of mechanisms of anesthetic action. We have been examining the effects of volatile anesthetics, such as isoflurane, on the nicotinic ACh receptor channel, to test models of direct and indirect interactions between small hydrophobic molecules and large membrane proteins. Isoflurane is a halogenated ether that is widely used as an inhalational general anesthetic for humans.

In a previous study of the actions of isoflurane on ACh receptor channels (3), we showed that the anesthetic decreased both the mean open time and burst duration and increased the number of gaps per burst and the gap duration. Those results were qualitative, in the sense that we had little control over the concentration of isoflurane reaching the patch. Nevertheless, we were able to test the adequacy of the sequential open-channel blocking model (4–8) in describing the effects of isoflurane. Although the sequential open-channel blocking model could explain our observations of a reduction in mean open time and the flickery appearance of channel events, it failed to explain the shorter burst durations found in the presence of isoflurane. The present study was undertaken in order to obtain quantitative concentration-effect relationships for the effects of isoflurane on channel kinetics and to use these relationships to test models of the action of isoflurane.

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[1]Deceased.

ABBREVIATIONS: ACh, acetylcholine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Materials and Methods

Nicotinic ACh receptors expressed by the clonal BC3H-1 cell line (9) were studied using single-channel recording techniques. Cells were cultured and prepared for electrophysiological experiments (10). Culture medium was replaced with an "extracellular solution" containing (in mM) NaCl, 150; KCl, 5.6; $CaCl_2$, 1.8; $MgCl_2$, 1.0; and HEPES, 10; pH 7.3. Patch pipettes were filled with a solution consisting of (in mM) KCl, 140; EGTA, 5; $MgCl_2$, 5; and HEPES, 10; pH 7.3; they had resistances of 4–6 MΩ. An outside-out patch (11) with a seal resistance of 10 GΩ or greater was obtained and moved into position at the outflow limb of the perfusion system. This perfusion system consisted of two reservoirs (plastic intravenous drip bags) containing 200 nM ACh in extracellular solution, a manual switching valve, and plastic (ethylvinyl acetate) tubing, one end of which was immersed in the culture dish. The desired mixture (0.175–4.0%) of isoflurane (1-chloro-2,2,2-trifluroethyl difluromethyl ether) (Forane; Anaquest, Madison, WI) in air was supplied by a calibrated isoflurane vaporizer and bubbled (flow rate, 1 liter/min) into one of the reservoirs. Excess anesthetic vapor was safely exhausted out of the laboratory. Bubbling of the solution was begun at least 20 min before data acquisition, a time we found sufficient for equilibration of vaporized isoflurane and extracellular solution. Bubbling was continued for the duration of the experiment.

Single-channel currents were measured with a patch-clamp amplifier (EPC-7; List Electronic, Darmstadt, FRG), filtered at 3 kHz (−3 db frequency, eight-pole Bessel filter; Frequency Devices, Haverhill, MA), digitized at 50 μsec/point, and stored on the hard disk of a laboratory computer (Micro 11/73; Digital Electronics Corp., Maynard, MA). Data analysis was performed off-line with the aid of our own computer programs.

The protocol for data collection was to record 4–10-s segments of single-channel activity with control (no anesthetic) solution flowing past the excised patch. Data segments were obtained while the patch was being held at several voltages between +100 and −130 mV. Next, the valve was switched to allow anesthetic-containing solution to flow past the patch; about 20 sec were needed for the new solution to reach the patch. Data collection was then repeated in the presence of anesthetic. Finally, recovery from the effects of the anesthetic was examined by returning to control solution and obtaining another set of data. The sequence was repeated until the demise of the patch.

Usually, the protocol sequence was repeated two or three times with similar results. Occasionally, there was evidence for rundown of the control behavior, that is, a gradual reduction in the frequency of openings, sometimes accompanied by a shorter open time. This shorter open duration was probably not due to residual anesthetic near the patch, because other channel properties (duration of brief gaps and single-channel conductance) were similar to their values in control. Moreover, we and others (12) have observed rundown in open time in excised patches never exposed to anesthetics. The results presented here were limited to experiments for which the rundown in open time was <20%.

Selected traces of digitized channel data containing relatively long duration openings were used to assemble an initial amplitude histogram. An estimate of the single-channel current was obtained from the difference in current between the base-line peak and the peak corresponding to one channel open. The entire segment of raw data was then examined. An opening transition was assumed to take place when two or more consecutive digitized points were above a threshold value of one half the estimated single-channel current above base line. Closing transitions were assumed to occur when at least one digitized point fell below the threshold current. Thus, the resolution was 100 μsec for openings and 50 μsec for closures. A second assessment of the open duration was made by using a threshold of one half of the calculated amplitude of that particular event. This approach was found to improve the fitting of single-channel events when rapid flickering was present. One of the authors or a trained assistant could then fine-tune the base line or reject intervals containing spurious noise.

Histograms of closed, open, and burst durations were constructed and analyzed as described previously (3). Two criteria were used to determine the critical gap time needed to define a burst (13, 14). Although the values of the critical gap time obtained from the two methods differed when the interburst interval was short, only slight differences in the characteristics of bursts (burst duration and number of openings per burst) were found. The critical gap time determined by the method of Colquhoun and Sakmann (13) was used for the data presented here.

Open-time histograms derived from control recordings were best fit by a two-component probability density function. The channel conductances of the two components were indistinguishable. The brief component had a time constant about 10 times shorter than the long component and accounted for 20–30% of the total number of openings at and above 200 nM ACh (Fig. 1). The time constant of the longer component was about 20% larger than the mean duration of all openings. Only one component was seen in the open time distributions derived from isoflurane experiments. In fitting the isoflurane concentration dependence of the open time to models, we used the mean open times and mean burst durations even for control experiments (see Discussion). The open time and number of openings per burst were corrected for missed gaps (13). This correction resulted in shorter open times (average, 10% shorter; range, 2–20%) and greater numbers of openings per burst (average, 10% increase; range, 2–24%) than before correction. Single-channel current amplitude histograms were constructed from only those openings that lasted longer than 0.2–0.3 msec.

Experiments were performed at room temperature (20–23°). The data are from 20 patches; between two and five patches were examined at each concentration. For each patch, single-channel recordings were made in the absence of isoflurane and at one isoflurane concentration. For each experimental condition, between 100 and 7000 events were accumulated for histogram analysis of event durations. Average values presented in the text and shown in figures include standard deviations. Isoflurane concentrations are expressed as both percentage of atmospheric pressure and millimoles in aqueous solution, based on a Bunsen water/gas partition coefficient of 1.08 at 25° (15). The anesthetic potency of isoflurane is about 1% (0.5 mM) (15).

Results

Isoflurane causes nicotinic ACh receptor channels to flicker. This effect was apparent as the data were collected and can be seen in Fig. 2. Whereas the normal pattern of channel behavior at low agonist concentrations consists of isolated openings

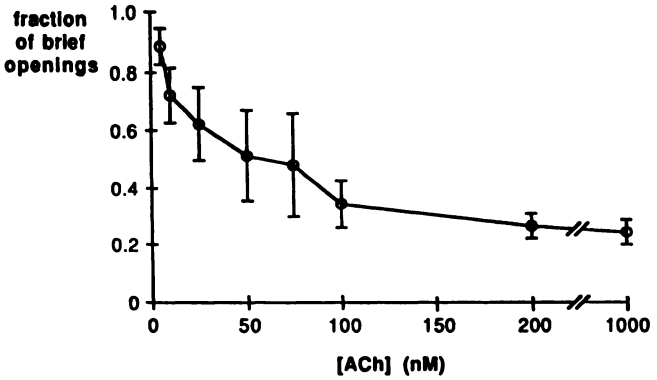


Fig. 1. Fraction of brief channel openings as a function of the ACh concentration. Open duration histograms were fit to a two-exponential probability distribution function, corrected for missed events. The fraction of brief openings is the amplitude of the faster (0.24 ± 0.10 msec) component. Each *point* represents the mean and standard deviation of the results from experiments on 2–12 patches, containing 200–2000 openings each. Applied potential was −100 mV. The remainder of the single-channel experiments described in this paper were done using 200 nM ACh, to obtain the smallest fraction of brief openings while keeping the channel activity relatively low.

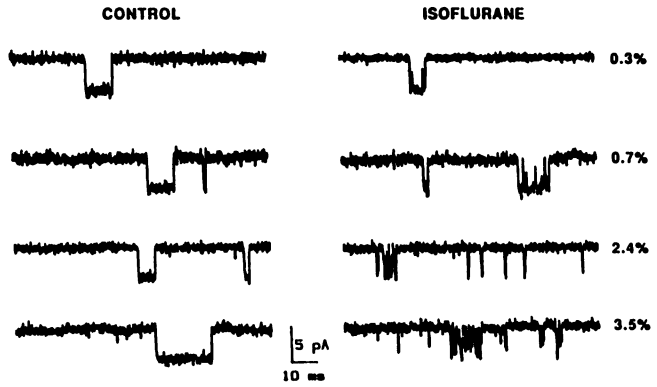


Fig. 2. Current recordings from outside-out patches of BC3H-1 cells, illustrating the effects of isoflurane on single ACh receptor channels. Each pair of 100-msec traces was obtained from the same patch before (*left*) and during (*right*) perfusion of the indicated concentration of isoflurane. Patch potential, −100 mV; 200 nM ACh; 3-kHz filter.

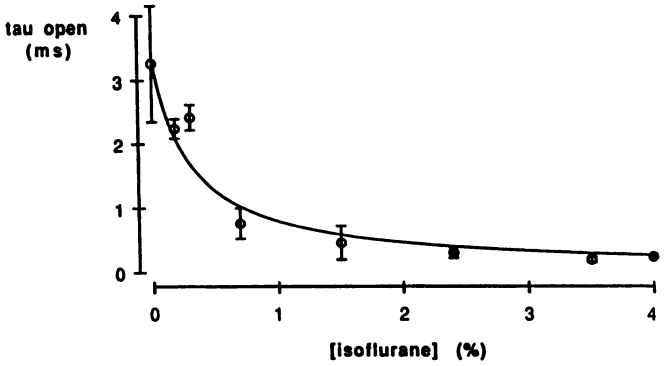


Fig. 3. Isoflurane concentration dependence of the mean open time (time constant of single exponential fit to open duration histogram) of ACh receptor channels. Data points represent the mean and standard deviation of measurements on two to five patches at each concentration. The *solid line* is the best fit to both the sequential (Scheme I) and open/closed (Scheme II) channel blocking models (eq. 1), with α = 310/sec and f = 1000/%/sec. Patch potential, −100 mV; 200 nM ACh.

occasionally interrupted by brief closures,[2] in the presence of the anesthetic, channel activity occurs in bursts of short openings separated by frequent brief closures. The effects of isoflurane are reversible; the normal pattern of channel behavior resumes within seconds of returning to control solution.

The dependence of kinetics of ACh receptor channels at −100 mV on the concentration of isoflurane is presented in Figs. 3–7. Isoflurane causes a dose-dependent reduction in the mean channel open time (Fig. 3). The open time is reduced by half at between 0.3% and 0.7% (0.14 and 0.34 mM) isoflurane and is less than one tenth of the control value at concentrations greater than 2.4% (1.2 mM). In the presence of the anesthetic, individual channel openings occur in bursts. The mean duration of bursts decreases as a function of isoflurane concentration (Fig. 4). In control experiments, the duration of bursts is only slightly greater than the duration of single openings, because brief closures are rare. At the highest concentrations of isoflurane, the burst duration is 2–3 times longer than the mean open time.

[2]Outside-out patches from BC3H-1 cells typically contain 50–150 nicotinic ACh receptor channels, but the channel open probability at 200 nM ACh is $<10^{-3}$ (36). Thus, openings separated by more than a few milliseconds probably arise from two different channels in the patch.

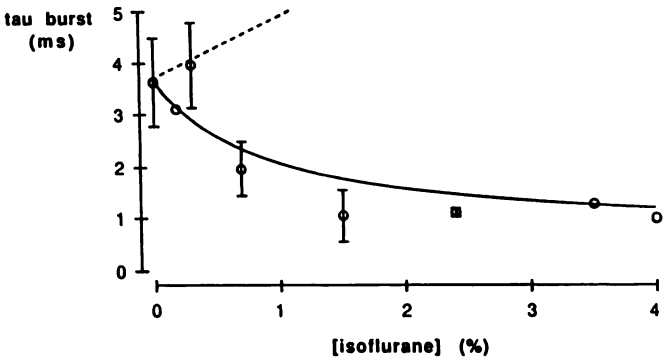


Fig. 4. Dependence of burst durations on isoflurane concentration. The *dashed line* corresponds to the prediction of the sequential channel block model (Scheme I, eq. 4, with b = 2000/sec). The *solid line* is the prediction of Scheme II (eq. 5, with b = 2000/sec). See legend to Fig. 3 for more details.

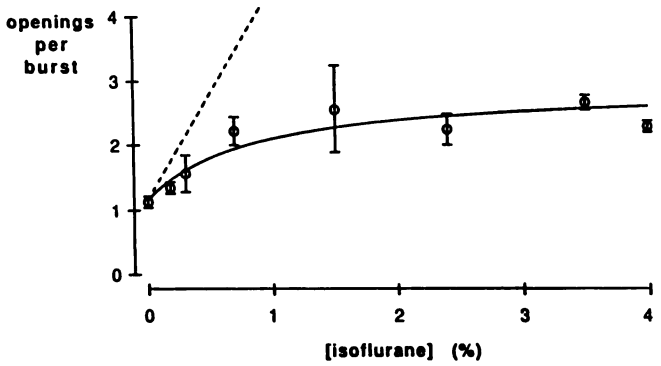


Fig. 5. Number of openings per burst as a function of isoflurane concentration. The *dashed line* corresponds to the prediction of the sequential channel block model (Scheme I, eq. 4). The *solid line* is the best fit of the data to the open/closed channel block model (Scheme II, eq. 7, with b = 2000/sec and α' = 1100/sec). See legend to Fig. 3 for more details.

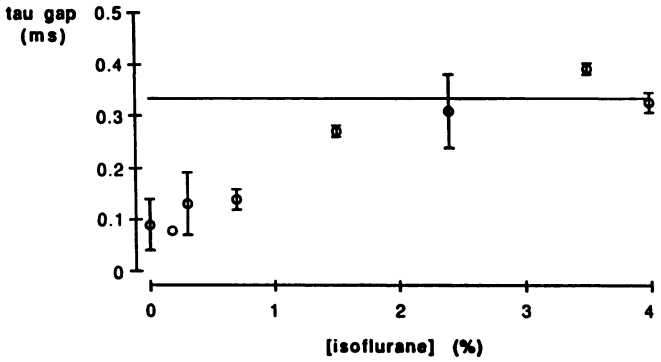


Fig. 6. Duration of gaps within bursts as a function of isoflurane concentration. The *solid line* corresponds to the assumption that the gaps induced by isoflurane are 320 μsec, independent of concentration. The gaps seen at low drug concentrations are a mixture of normal gaps and isoflurane-induced gaps. See legend to Fig. 3 for more details.

A quantitative measure of channel flicker induced by isoflurane is the average number of openings in a burst (Fig. 5). This increases from about 1.14 ± 0.09 (mean ± standard deviation, n = 20) in control records to >2.5 at high anesthetic concentrations. Because each burst consists of at least one opening, a better illustration of the dramatic effect of isoflurane on channel flicker is obtained by comparing the number of gaps per burst. The effect of 3.5% (1.7 mM) isoflurane is to increase the number of gaps per burst 10-fold.

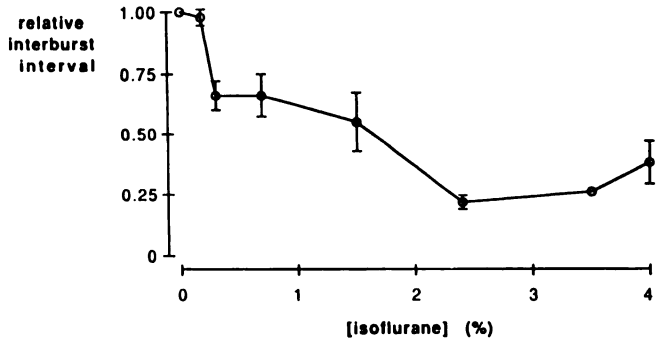


Fig. 7. Isoflurane concentration dependence of the interburst interval. Each *point* represents the mean and standard error of the time between bursts in the presence of isoflurane, relative to that in control. See legend to Fig. 3 for more details.

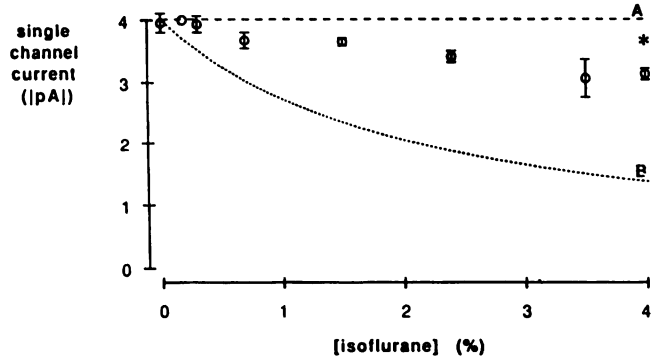


Fig. 8. Absolute value of the single-channel current at −100 mV as a function of isoflurane concentration. *Line A* corresponds to the prediction of either model with the assumption that all brief gaps are resolved. *Line B* is the single-channel current, i, that would be measured if no gaps could be resolved, $i = i_0/(1 + f[B]/b)$, where $i_0 = 4$ pA is the single-channel current in the absence of isoflurane. The *asterisk* at 4% isoflurane was obtained from analysis of filtered simulated data (Scheme II), to estimate the effect of missed gaps on our measurement of single-channel current. See legend to Fig. 3 for more details.

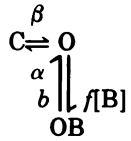
The duration of the brief gaps seen in bursts of channel activity is shown in Fig. 6. The infrequent gaps that occur in the absence of anesthetics have a mean duration between 50 and 100 μsec. These gaps are thought to arise from brief closures of a single channel followed by reopening before agonist dissociation and are more conveniently studied at lower temperatures (13, 16). As the concentration of isoflurane is increased, not only do gaps become more numerous, they also have a longer duration. The duration appears to saturate near 300–400 μsec at high anesthetic concentrations.

Isoflurane increases the frequency of occurrence of bursts. This is seen in Fig. 7, which shows a concentration-dependent decrease in the mean interburst interval, $\tau_{interburst}$. Because the frequency of bursts varies from patch to patch, due to differences in the number of channels in the patch (range, 2–80/sec), the interburst interval in the presence of isoflurane is normalized to the control value.

We also examined the effect of isoflurane on ACh receptor single-channel current at −100 mV (Fig. 8) and conductance. Conductances were obtained from the slope of single-channel current-voltage curves over the range of −130 to 100 mV. In the absence of isoflurane, the conductance of the ACh receptor channel is 40 ± 3 pS. At 4% (1.9 mM) isoflurane, the apparent conductance is 25% lower. At all concentrations of isoflurane, the current-voltage curves are linear, with reversal potentials close to 0 mV.

Previously (13), we tested the idea that the flickering behavior of ACh receptor channels exposed to isoflurane arose from transient blockages of open channels by isoflurane. Although the perfusion system used in those experiments could not deliver a known concentration of isoflurane to the cell-attached membrane patch, we showed that a sequential open-channel blocking model could not explain some of the actions of isoflurane. The data presented in this paper were obtained under well controlled anesthetic concentrations and warrant another assessment of the channel block as a mechanism for the action of isolflurane.

The sequential open-channel blocking model (6) is diagrammed in Scheme I.



Scheme 1

In this model, a channel may be in one of three conformational states, closed (C), open (O), or blocked while the gate is open (OB). In the case of the nicotinic ACh receptor, the closed state is a composite of unliganded and liganded closed states that are not distinguished in experiments performed at low agonist concentrations. The transition from this composite state to the open state takes place at a rate β (a function of binding, unbinding, and opening rates). The open channel can either close to C at a rate α or be blocked at a rate $f[B]$, the product of the forward blocking constant f and the concentration of the blocker [B]. The model assumes that the blocker must leave the channel (dissociation rate b) before the channel can close.

This model makes quantitative predictions about the kinetic behavior of single channels, i.e., mean open time, τ_{open}, burst duration, τ_{burst}, gap duration, τ_{gap}, and the number of openings per burst, $N_{o/b}$ (17).

$$\tau_{open} = \frac{1}{\alpha + f[B]} \quad (1)$$

$$\tau_{burst} = \frac{1 + f[B]/b}{\alpha} \quad (2)$$

$$\tau_{gap} = \frac{1}{b} \quad (3)$$

$$N_{o/b} = \frac{\alpha + f[B]}{\alpha} \quad (4)$$

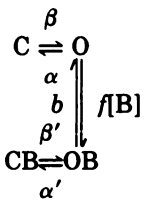
Implicit in these equations is the assumption that the dissociation rate is slow enough to allow gaps to be resolved. An additional prediction under this assumption is that single-channel conductance is unaffected by the blocker. In this model, the blocker has no effect on the interburst interval.

One feature of the action of isoflurane on the ACh receptor channel that is clearly consistent with sequential open channel block is the reduction in mean open time. Not only is the mean open time reduced in the presence of the anesthetic, but the functional dependence of the mean open time on isoflurane concentration is in quantitative agreement with the model. This is shown in Fig. 3, where the fit to eq. 1 is drawn as a solid line. The fit is obtained with only one adjustable parameter, f, the forward blocking rate constant, because the channel

closing rate, α, is simply the reciprocal of the open time in control records. The line superimposed on the data in Fig. 3 is for $\alpha = 310$/sec and $f = 1000$/%/sec (2.1×10^6/M/sec).

In agreement with the sequential open-channel block model, the number of openings per burst increases with the concentration of isoflurane. However, using the aforementioned values of α and f in eq. 4, we find a quantitative difference between the prediction (Fig. 5, *dashed line*) and our observations. Isoflurane induces substantially fewer gaps than expected from its apparent forward blocking constant. Moreover, Scheme I fails to predict even the qualitative dependence of burst duration on isoflurane concentration. Because blocking events can only prolong the time to channel closure, the burst duration should increase, rather than decrease, as a function of blocker concentration.

In spite of the unsatisfactory performance of the sequential open-channel blocking model (Scheme I) in predicting the features of bursts seen with isoflurane, the ability of eq. 1 to describe the concentration dependence of τ_{open} so well led us to think that some extension of the model, rather than an entirely different kinetic scheme, would be the logical next step in describing the actions of isoflurane. What is needed is a means of terminating the burst earlier than predicted by sequential block. Thus, a model in which blocked channels can close and do so at a rate faster than the normal closing rate ought to give at least qualitative agreement with the burst data. This model is depicted as Scheme II.



Scheme II

Here, open but blocked channels (OB) can close while the blocker is still bound (state CB). If we define a burst as the time spent in either the open or open/blocked state (the same definition used in Scheme I), the closing rate, α', becomes the only additional free parameter (the blocked channel opening rate β' is shown for completeness; see Discussion). Using methods outlined by Colquhoun and Hawkes (18), we derived expressions analogous to eq. 1–4 for the kinetic measurements obtained from single-channel records. Because Scheme II contains only one open (conducting) state, the expression for the mean open time, τ_{open}, is identical to eq. 1. The blocked-channel closing rate, α', appears in the other expressions.

$$\tau_{burst} = \frac{1 + f[B]b/(b + \alpha')^2}{\alpha + f[B]\alpha'/(b + \alpha')} \quad (5)$$

$$\tau_{gap} = \frac{1}{b + \alpha'} \quad (6)$$

$$N_{o/b} = \frac{\alpha + f[B]}{\alpha + f[B]\alpha'/(b + \alpha')} \quad (7)$$

In Scheme II, the duration of brief gaps is predicted to be independent of the concentration of the blocker (eq. 6). Although the observed relationship is not concentration independent, a reasonable interpretation of Fig. 6 is that the observed gaps originate from two sources, with those gaps seen with ACh alone and gaps arising from blocked to closed transitions appearing as a single component under our recording conditions. Then, as the concentration of blocker is increased, the fraction of blocking gaps should increase and the observed gap duration should saturate. From Fig. 6, it appears that, at concentrations of isoflurane at or above 1.5% (0.72 mM), the gap duration saturates at about 0.32 msec. With this assumption, we determine $b + \alpha'$ from the limiting value of τ_{gap}. Inspection of eq. 7 reveals that the number of openings per burst depends on b/α', not b or α' separately. For our data, $b + \alpha' = 3100$/sec and $b/\alpha' = 1.7$, which gives $b = 2000$/sec and $\alpha' = 1100$/sec. Using these values in eqs. 5 and 7 results in the solid curves in Figs. 4 and 5. Thus, Scheme II gives a satisfactory fit to the concentration dependence of the properties of bursts induced by isoflurane, with the addition of only one adjustable parameter to the sequential block model.[3]

If every gap induced by the blocker is resolved, Scheme II predicts that the single-channel conductance is independent of concentration. However, for isoflurane the mean duration of gaps is only 320 μsec; some gaps will be missed due to filtering and digitization. Unresolved gaps will tend to decrease the apparent single-channel conductance. We estimate that about 50% of the observed decrease in conductance can be accounted for by unresolved gaps (see Discussion). Scheme II makes no prediction about the concentration dependence of the interburst interval (see Discussion).

We also examined single-channel data obtained at +100 mV (over a more limited concentration range) and fit these data to Scheme II. In Table 1 we compare the four kinetic parameters of Scheme II at positive and negative potentials. The channel closing rate α, which has previously been shown to be voltage dependent (13, 16, 19), exhibits the largest voltage dependence. The forward blocking rate constant is also somewhat faster at depolarized potentials. The other parameters, α' and b, are relatively insensitive to voltage.

Discussion

Scheme II, an extension of the sequential open-channel blocking model that allows for unblocking of closed channels, is consistent with many features of the actions of isoflurane on single ACh receptor channels. Others have suggested that some form of closed channel block might be an appropriate model for the action of drugs on neuromuscular nicotinic ACh receptor channels, based on measurements of end-plate currents (6, 7, 20–22) and single channels (23–26). Quantitative analysis of end-plate currents (20, 21) supports such a model for the actions of alcohols and general anesthetics. Papke and Oswald (26)

TABLE 1

Comparison of the fitted parameters to Scheme II at two voltages

	−100 mV	+100 mV	+100/−100*
α (1/sec)	310	1000	3.1
f (1/M/sec)	2.1×10^6	5.2×10^6	2.5
b (1/sec)	2000	1300	0.6
α' (1/sec)	1100	1200	1.1

* The ratio of the parameter value at +100 mV to that at −100 mV.

[3]The burst duration and number of openings per burst are not independent parameters because, by definition, $\tau_{burst} = N_{o/b}\,\tau_{open} + (N_{o/b} - 1)\,\tau_{gap}$. Agreement of Scheme II with both the burst duration (Fig. 4) and number of openings per burst (Fig. 5) data does not constitute separate lines of evidence in support of the model. An alternative description is the frequency of gaps per unit open time, introduced by Ogden and Colquhoun (24). In terms of Scheme II, this frequency is given by $f[B]b/(b+\alpha')$.

found that the action of tetracaine on single channels could be interpreted in terms of open- and closed-channel block. Ogden and Colquhoun (24) considered a variety of schemes in their study of channel block by suberyldicholine and concluded that sequential block was sufficient to explain their observations.

Scheme II differs from sequential open-channel block (Scheme I) by only one additional state (CB) and rate constant (α'). Nevertheless, the scheme is quite flexible; a value for b/α' in eq. 7 can be found to fit nearly any monotonic saturating change in the number of openings per burst as a function of concentration. Moreover, its application here depends on our assumption about the concentration dependence of the gap duration (Fig. 6). Clearly, other evidence to support the model is necessary. One approach would be to make independent determinations of some of the rate constants in the model. A subsequent paper[4] will present evidence supporting Scheme II, based on results of rapid drug perfusion experiments.

The blocking rate constant obtained for isoflurane, 2.1×10^6/M/sec, is smaller than what would be expected for a diffusion-limited process. It is also smaller than the blocking rates obtained for cations that are thought to block open channels by binding to the channel lumen such as ACh (4×10^7/M/sec) (24, 27) and QX222 (2×10^7/M/sec) (28). The binding of these cations is voltage dependent; Neher and Steinbach (28) extrapolated the association rate constant for QX222 to zero applied potential to get 4×10^6/M/sec. Application of the sequential channel blocking model to the actions of other molecules on the ACh receptor, i.e., barbiturates (6), benzocaine (29), hexanol, heptanol, and octanol (30), and ketamine (31), gave blocking rate constants in the range of 10^6 to 10^7/M/sec. If these molecules and isoflurane do indeed bind to the ACh receptor-ion channel complex, the binding site may or may not be the channel lumen. There are no assumptions about the location of the binding site inherent in kinetic models such as Schemes I and II.

Scheme II does not provide a complete description of the effects of isoflurane on the ACh receptor. For one thing, there is the apparent decrease in single-channel current shown in Fig. 8. To determine the extent of this decrease that may arise from unresolved gaps, we made single-channel simulations of Scheme II for 4% isoflurane, added noise and 3-kHz filtering, and analyzed these artificial channel records just as we would real data. The resulting single-channel current is shown as an asterisk in Fig. 8. Limited resolution may account for about half of the measured reduction in single-channel current, but we cannot exclude the possibility that isoflurane has an additional effect on the channel that further attenuates the current.

Scheme II also does not deal with the changes in interburst interval seen with isoflurane (Fig. 7). The scheme could be generalized further to include pathways for the blocker to bind and unbind closed channels, both liganded and unliganded (transitions between C and CB). The increase in the number of bursts seen with isoflurane might then be attributed to a larger apparent opening rate for drug-bound channels ($\beta' > \beta$). Experiments performed with low concentrations of agonist cannot be used to distinguish changes in agonist binding affinity from changes in channel opening rate. The increase in burst frequency will be considered further in a subsequent paper,[4] in which the results of experiments using rapid perfusion of high agonist concentrations will be presented.

A complicating feature of ACh receptor channel kinetics is the presence of two components in open and burst duration histograms. Although some brief openings may arise from the opening of singly liganded receptors (32), this cannot be the origin of those seen at 200 nM ACh and higher concentrations (13, 33) (Fig. 1). Only the long duration component of bursts appears to be interrupted by brief gaps under control conditions (13, 16), so the brief burst component is often excluded from kinetic analyses. We might ask whether isoflurane preferentially affects long or short bursts. Although the open duration histogram collapses to a single exponential component when isoflurane is used, we often detect two burst components in these data. The duration of brief bursts in the presence of isoflurane (0.18 ± 0.14 msec) did not differ from its control value (0.24 ± 0.14 msec). Therefore, we examined our data considering only the long component in the analysis of bursts. The qualitative features of the effects of isoflurane remain unchanged; bursts become shorter and the number of openings per burst increases less steeply than predicted by the sequential channel block model. Quantitatively, we found the following values for the parameters in Scheme II: $\alpha = 230$/sec, $f = 1.9 \times 10^6$/M/sec, $b = 2400$/sec, and $\alpha' = 950$/sec. These values are not significantly different from those in Table 1 (−100 mV), for which all bursts were considered.

In our previous study (3), a pipette containing isoflurane dissolved in extracellular solution was positioned close to a cell-attached patch. The membrane area surrounding the patch was perfused with the isoflurane solution by applying pressure to the interior of the pipette. The fact that the effects of isoflurane were seen with this application system implies that the anesthetic can (but does not necessarily have to) use a membrane pathway to get to its site of action. The concentration of anesthetic at the channels underneath the patch pipette may be considerably less than the concentration in the pressure-injection pipette. Application of 5 mM isoflurane from the pressure-injection pipette reduces the mean open time to about 10% of control. The same reduction in open time is achieved by applying 2% (1 mM) directly to an excised patch (Fig. 3).

Sokoll *et al.* (34) examined the effects of isoflurane on miniature end-plate currents from frog muscle. They found that the decay time constant decreased linearly with the concentration of isoflurane; the time constant was reduced by a factor of 2 at 0.34 mM isoflurane. The decay rate is considered to be a measure of the channel closing rate, α. We found open times decreased by half at 0.18 mM, but the concentration dependence is nonlinear (Fig. 3). Sokoll *et al.* (34) also measured miniature end-plate current amplitudes and found a linear decrease with concentration. There is no direct way to compare this result with our data.

Our data provide evidence in favor of a direct interaction between isoflurane and the nicotinic ACh receptor protein. The concentration dependence of the channel open time (Fig. 3) is quantitatively consistent with an anesthetic-induced emergence of a new closed state adjacent to the open state (eq. 1). In contrast, purely lipid-based theories of anesthetic action assume that kinetic rate constants are modified by the drug while the number of states remains the same. It would be fortuitous if a change in the physical-chemical properties of the lipid bilayer would result in an increase in the channel closing

[4] J. P. Dilger and R. S. Brett. Effects of isoflurane on acetylcholine receptor channels. 2. Currents elicited by rapid perfusion of acetylcholine. Manuscript in preparation.

rate that mimicked the observed concentration dependence of channel open time. It is also difficult for lipid-based hypotheses of anesthetic action to account for the properties of bursts seen with isoflurane. Although flickering could arise from an increase in the channel opening rate or a decrease in the agonist dissociation rate, either of these changes would result in a lengthening of the burst duration, the opposite of what is observed (Fig. 4).

Patchclamp recording provides the opportunity to "see" not only individual ion channels in cell membranes but also interactions between single drug molecules and single ion channels. When single-channel techniques were used in combination with the new tools of molecular biology, local anesthetic binding sites were localized to a short segment of a transmembrane helix of the ACh receptor (35). If, as we suggest, general anesthetics also bind directly to the ACh receptor protein, the same tools should be able to confirm this suggestion and help reveal the long elusive anesthetic binding sites.

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