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The Effects of Isoflurane on Acetylcholine Receptor Channels.: 2. Currents Elicited by Rapid Perfusion of Acetylcholine

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SUMMARY

We studied the effects of the volatile anesthetic isoflurane on nicotinic acetylcholine (ACh) receptor channels using a technique for rapid perfusion of ACh to outside-out patches. Channels were activated by ACh, at concentrations ranging from 1 μM to 10 mM, and the macroscopic current flowing through tens or hundreds of channels was measured. Isoflurane reduced the peak current response to saturating concentrations of ACh, increased the current decay rate due to desensitization, and decreased the rate of recovery from desensitization. The effect of isoflurane on peak currents was concentration dependent; at 2% isoflurane, the peak current was reduced by half. The effect of isoflurane on the peak current induced by nonsaturating concentrations of ACh was smaller. We measured the onset and recovery of current inhibition by isoflurane, by rapidly applying and removing isoflurane to the patch within 100 μsec. With 2% isoflurane, currents were inhibited with a time constant of 200–300 μsec and recovered with a time constant of 500–700 μsec. We interpreted our results in terms of a kinetic model in which isoflurane binds directly to both open and closed channels (not necessarily within the pore of the channel) and stops the flow of ions through open channels. This model provides a quantitative explanation for the kinetic and equilibrium effects of isoflurane on peak currents activated by saturating concentrations of ACh. Our data support the idea that the flickering effect of isoflurane on single ACh receptor channels is caused by rapid binding and dissociation of isoflurane to an inhibitory binding site on the protein. The effects of isoflurane on the apparent affinity of ACh and on desensitization are not predicted by the model. These effects may arise from the binding of isoflurane to other sites, not necessarily on the protein itself.

Clinical concentrations of many general anesthetics affect nicotinic ACh receptor channels (1–7). In the first paper of this series (6), we reported on the effects of isoflurane on single ACh receptor channels activated by a low concentration of ACh (200 nM). We found that isoflurane caused the channel activity to occur in short bursts of brief openings (flickering). This behavior could be explained by a kinetic scheme in which isoflurane binds to both the closed and open states of the channel protein; binding to the open state blocks the flow of ions through the pore and increases the rate at which the gate of the channel closes. In this model, flickering arises from repeated blockages of the open channel, with each blockage lasting an average of 300 μsec. Because isoflurane remains bound even after the gate of the channel closes, the duration of bursts is decreased by the anesthetic.

Single channel measurements at low agonist concentrations do not provide enough information to determine all of the rate constants in the kinetic scheme. The effect of isoflurane on agonist binding, channel opening, and receptor desensitization cannot be inferred from such data. Information on the binding and dissociation of isoflurane from the closed channel states is also not available from these measurements. Single channel measurements over a range of agonist concentrations provide information on agonist binding and agonist efficacy. However, receptor desensitization by high concentrations of agonist results in a complex pattern of single ACh receptor channel kinetics (8–10). In order to avoid the complicating features introduced by desensitization, we used a rapid perfusion technique to study channels in outside-out patches activated by high (>1 μM) concentrations of ACh (11–14). This approach allows us to determine the channel opening rate, the peak open channel probability (before desensitization), the time course of rapid desensitization, and recovery from desensitization. In addition, we are able to determine the time course of isoflurane binding and dissociation by making rapid changes in isoflurane concentration across an excised patch.

Methods

Nicotinic acetylcholine receptors expressed by the clonal BC3H-1 cell line (15) were cultured and prepared for electrophysiological ex-

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ABBREVIATIONS: ACh, acetylcholine.

periments (16). The saline solutions used were identical to those described in part 1 of this study (6). An outside-out patch (17) with a seal resistance of 10 GΩ or greater was obtained from a cell and moved into position at the outflow of the perfusion system. This perfusion system consisted of solution reservoirs, manual switching valves, a solenoid-driven pinch valve, and a V-shaped piece of tubing inserted into the culture dish as described elsewhere (13). One arm of the "V" contained extracellular solution without agonist (normal solution). The other arm contained extracellular solution with a known concentration of ACh (test solution). In the resting position of the pinch valve, normal solution perfused the patch. The solenoid was then triggered to stop the flow of normal solution and start the flow of test solution. After the patch was exposed to test solution for tens or hundreds of milliseconds, the pinch valve was returned to its resting position for several seconds. This system allows us to subject the patch to a series (10–60) of timed exposures to agonist-containing solution, minimizing the desensitizing effects of prolonged exposure to ACh. The system produces a rapid exchange of the solution bathing the patch; the solution exchange time is usually less than 500 μsec, but it is faster than 100 μsec in some patches (13). The desired mixture (0.5–4.0%) of isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Forane; Anaquest, Madison, WI) in air was supplied by a calibrated isoflurane vaporizer and bubbled into some of the reservoirs as described before (6). This range of partial pressures of isoflurane corresponds to an aqueous concentration range of 0.25–2 mM. The Bunsen water/gas partition coefficient for isoflurane is 1.08 at 25°.

The currents that flowed during exposure of the patch to ACh were measured with a patch clamp amplifier (EPC-7; List Electronic, Darmstadt, Germany), filtered at 3 or 10 kHz (−3 db frequency, 8-pole Bessel filter; Frequency Devices, Haverhill, MA), digitized at 200 or 10 μsec/point, and stored on the hard disk of a laboratory computer. Data analysis was performed off-line with the aid of our own computer programs.

The first step was to record the current responses (at −50 mV) to application of a test solution containing 100 μM ACh, a concentration that opens about 90% of the channels (12). This served as a reference point for estimating the number of channels in the patch. We returned to this test solution at various times during the life of the patch to determine whether there was any loss of channel activity. Both the normal and test perfusion solutions were switched to isoflurane-containing solutions by means of manual valves (about 30 sec are required to completely exchange the solutions in the "V" of the perfusion system). The response of the patch to 100 μM ACh during continuous exposure to isoflurane was then recorded. After returning to the reference solutions, we repeated the sequence with another concentration of ACh. This protocol was continued until the demise of the seal or loss of channel activity (anywhere from a few minutes to several hours). Any deviation from this protocol is noted in the text. Experiments were performed at room temperature (20–23°).

The ensemble mean current was calculated from between 10 and 60 individual current traces. Mean currents were fit to a single exponential to obtain peak and steady state current values and a time constant of the decay due to desensitization (14). When the sampling rate was 10 μsec/point, individual traces were aligned before calculation of the ensemble mean (13). Peak open channel probability, p_o, is calculated from the peak mean current, I_p, the number of channels in the patch, N, and the single channel current, i, from Eq. 1:

$$I_p = Nip_0 \quad (1)$$

Results

Fig. 1 illustrates the effect of isoflurane on the current response to application of 100 μM ACh. In the absence of isoflurane (unlabeled traces), the currents increase quickly to a peak current as ACh is applied and then decay in the continued presence of ACh (rapid desensitization). Isoflurane reduces the peak current in a concentration-dependent manner: the

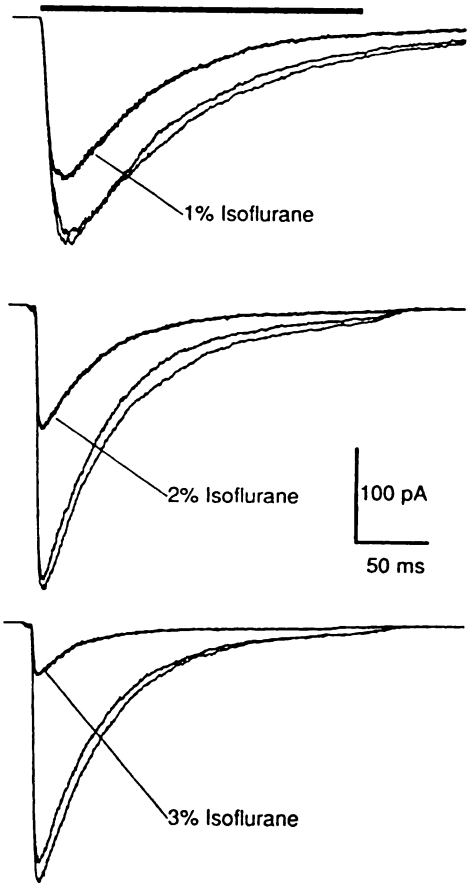


Fig. 1. The effects of 1, 2, and 3% isoflurane on ensemble mean currents obtained from rapid perfusion of 100 μM ACh onto an excised patch containing about 125 ACh receptor channels. ACh was perfused for 250 msec at 3-sec intervals. Each panel contains two control mean currents (unlabeled) and one mean current obtained while the patch was exposed to the indicated concentration of isoflurane. One control was obtained before exposure to isoflurane and the other control was obtained after exposure to isoflurane. Patch held at −50 mV. The horizontal bar in the top panel indicates the time of agonist perfusion.

peak currents are 0.75, 0.42, and 0.19 of the controls for 1, 2, and 3% isoflurane, respectively. Isoflurane also decreases the time constant of current decay, but this decrease is not strongly related to concentration: the time constants are 0.74, 0.83, and 0.63 times control for 1, 2, and 3% isoflurane, respectively. We found previously that in control recordings, the decay time constant is variable from patch to patch and sometimes during the lifetime of the patch (14). This is evident in Fig. 1, in which the control decay time constants in a single patch are 95, 50, and 47 msec in the three panels.

The results of experiments on 16 patches in which we examined the effect of isoflurane on peak currents elicited by 100 μM ACh are summarized in Fig. 2A. There is considerable variability in the relative peak currents from patch to patch. We have not identified the source of this variability; it may be that the concentration of isoflurane was not as well controlled during rapid perfusion as we had hoped. However, the trend toward lower peak currents is evident. In contrast, there is no correlation between the decrease in decay time constant and isoflurane concentration (Fig. 2B). The relative decay time constant averaged over all isoflurane concentrations was 0.67 ± 0.15 (37 determinations).

Recovery from desensitization (14) is also affected by isoflu-

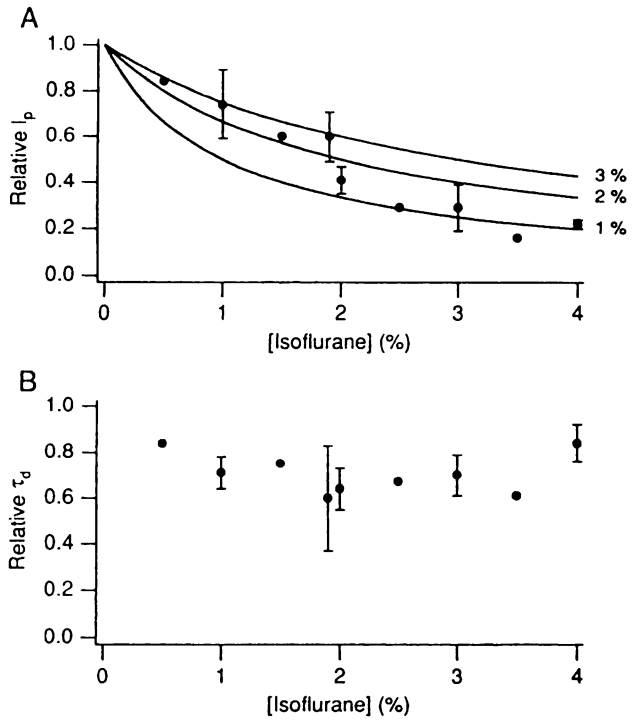


Fig. 2. A, The isoflurane concentration dependence of the decrease in the relative peak current response, I_p, to 100 μM ACh. The peak current in the presence of isoflurane is normalized to the peak current in control records. Each point represents the mean and standard deviation from two to 10 experiments. The continuous lines correspond to the predictions of Eq. 2 with the indicated values of b/f, the affinity of the isoflurane binding site. See Discussion for details. B, The relative decay time constant, τ_d, as a function of isoflurane concentration. The data come from the same experiments described above.

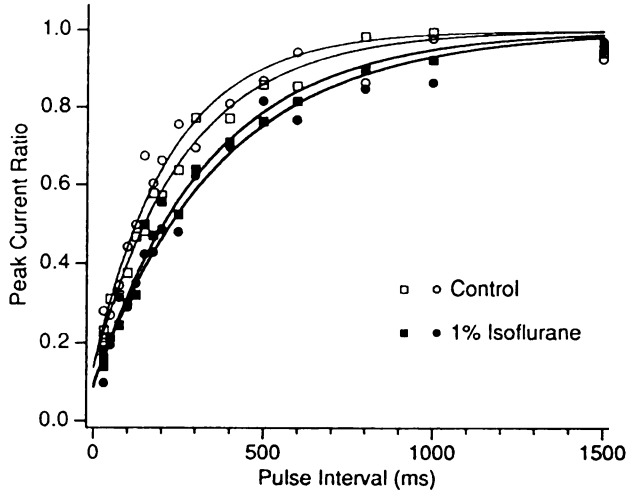


Fig. 3. Slowing of the recovery from desensitization by 1% isoflurane. A 250-msec application of 100 μM ACh was used to desensitize most of the channels. A second application of 100 μM ACh was made after a delay of 30–3000 msec (pulse interval). The peak current ratio is the peak current elicited by a second application relative to the first. The open symbols are the results of control runs made before (□) and after (○) two isoflurane runs (closed symbols). The lines are single exponential fits to the data with recovery time constants of 270 (□) and 230 msec (○) in control and 340 (■) and 380 msec (●) in the presence of 1% isoflurane.

rane. Fig. 3 shows recovery curves in control and with 1% isoflurane. Isoflurane reproducibly increases the time constant for recovery by almost 50%. We have obtained similar results at other concentrations of isoflurane.

We then considered the effects of isoflurane on peak currents elicited by different concentrations of ACh. Fig. 4 presents some examples in which a patch exposed to 1.9% isoflurane was perfused with 3, 10, 30, 100, and 300 μM ACh. Although isoflurane reduced the peak currents induced by the higher concentrations of ACh, it had considerably less effect on the 10 μM and 3 μM ACh currents. The relative peak currents for this patch were 0.66, 0.75, 0.67, 0.95, and 1.0 for 300, 100, 30, 10, and 3 μM ACh, respectively.

The results from four patches are summarized in Fig. 5 as the peak open channel probability as a function of ACh concentration. The reduction in peak open probability by 1.9% isoflurane is most pronounced at higher agonist concentrations. When 1 μM ACh was applied, the peak open probability was actually increased by 25% by 1.9% isoflurane (average of two patches, individual open probabilities were 1.08 and 1.47 times control).

Some patches exhibit an extremely rapid solution exchange and we were able to use such patches to determine the kinetics of the action of isoflurane. Fast patches can be identified by perfusing 10 mM ACh at −50 mV (13). Under these conditions, ACh quickly and completely saturates the receptor binding sites that have an affinity of 60 μM. But 10 mM ACh also acts like a fast open channel blocker and reduces the peak current by about 50%. If solution exchange is slow, the evoked current exhibits a decay as the ACh concentration rises to the blocking range (this decay occurs on the millisecond time scale and is considerably faster than the decay due to desensitization). If solution exchange is fast, the current is at its blocked level throughout the response, and no decay is seen. Fig. 6A shows control currents at high time resolution in one such fast patch held at +50 mV (large trace in upper panel) and −50 mV (large trace in lower panel). The currents activate with a 20–80% time of 40 μsec. At +50 mV, this represents the opening isomerization of the channel [at −50 mV, it is difficult to interpret the activation time in terms of channel opening (13)]. The current at +50 mV is larger than the current at −50 mV because 10 mM ACh is not an effective blocking concentration at +50 mV.

Fig. 6A also shows currents activated by 10 mM ACh in the continuous presence of 2% isoflurane, i.e., both the normal and test perfusion solutions contain the anesthetic, as in our normal protocol. Isoflurane inhibits the current to 45% (+50 mV) or 60% (−50 mV) of control nearly uniformly over the duration of agonist application. We will refer to this level of current as the equilibrium level.

Fig. 6B illustrates what happens when isoflurane is rapidly perfused to the patch along with ACh. In this protocol, the test solution contained isoflurane but the normal solution did not. The current initially rises to nearly the control level and then decays to the equilibrium level for 2% isoflurane. The decay occurs with a time constant of 330 μsec at +50 mV and 310 μsec at −50 mV. This is considerably slower than the solution exchange time (<100 μsec in this patch). Thus, the decay represents the time required for isoflurane to equilibrate at the inhibitory site(s) and inhibit the current.

Fig. 6C shows the effect of rapid removal of isoflurane from the patch. In this protocol, isoflurane was present in the normal

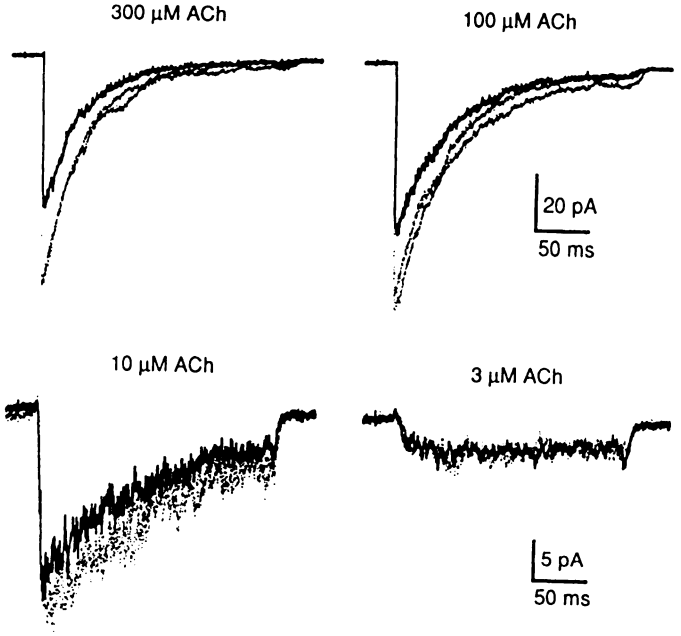


Fig. 4. The effect of 1.9% isoflurane on the ensemble mean currents obtained from rapid perfusion of different concentrations of ACh. All data are from a single patch containing about 50 channels. Each panel contains two control mean currents (dotted traces, before and after exposure to isoflurane) and one mean current obtained while the patch was exposed to 1.9% isoflurane (continuous traces). Note that both bottom panels (10 and 3 μM ACh) are plotted on an expanded current scale.

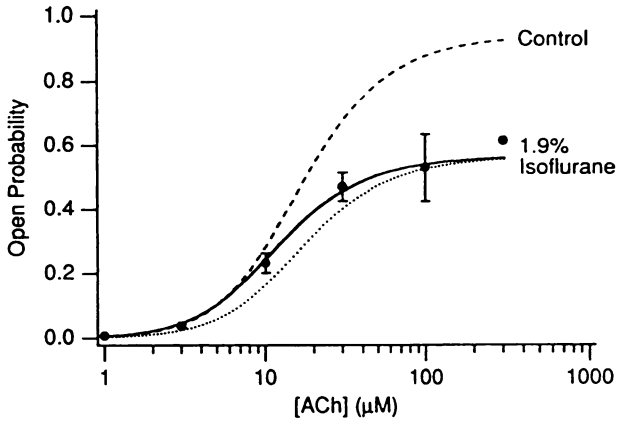
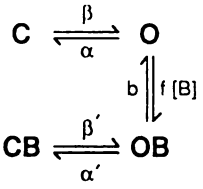
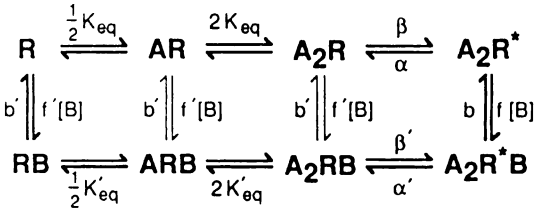


Fig. 5. Concentration-response curves for activation of ACh receptor channels by ACh in the absence and presence of 1.9% isoflurane. The symbols represent the average and standard deviations of the peak open probability in the presence of isoflurane with four patches. The control curve (dashed line) is drawn according to Eq. 4 with [B] = 0, K_{eq} = 56 μM, β/α = 17 (12). The dotted line near the isoflurane data is for [B] = 1.9% and the same equilibrium parameters. The solid line through the isoflurane data is for [B] = 1.9%, K_{eq} = 40 μM, β/α = 17.



Scheme 1



Scheme 2

solution but not in the test solution. Here, the current is initially at the equilibrium level for 2% isoflurane and recovers to the control level with a time constant of 680 μsec at +50 mV and 750 μsec at −50 mV. Recovery is not limited by the speed of solution exchange. It reflects the time needed for isoflurane to leave the inhibitory site(s) and for the channel to recover from exposure to isoflurane.

Table 1 summarizes the results of onset and recovery experiments like the one shown in Fig. 6 on four patches with 2% isoflurane. At both +50 and −50 mV, the onset of inhibition by isoflurane is faster than the recovery from inhibition by the anesthetic.

Close examination of Fig. 6 reveals that the equilibrium current trace is not completely constant but exhibits a brief overshoot and undershoot. These effects occur in most, but not all, of our onset and recovery experiments. We have not yet determined the cause of these effects. Fig. 7 shows an example in which the equilibrium current trace is flat. When the two control and one equilibrium current traces are scaled to have the same amplitude, they appear to be identical. All three traces have a 20–80% rise time of 50 μsec. In this patch, the onset and recovery traces (not shown) exhibit the same sort of decays seen in Fig. 6.

Discussion

In our earlier paper (6), we presented support for Scheme 1 as the kinetic mechanism of action for isoflurane. In this model, isoflurane is assumed to bind to both the closed state (C) and the open state (O) of the channel and form two distinct, nonconducting "blocked" states CB and OB. The use of the term blocked in this discussion does not necessarily imply that isoflurane inhibits currents by physically obstructing the pore of the channel; isoflurane may bind elsewhere on the protein

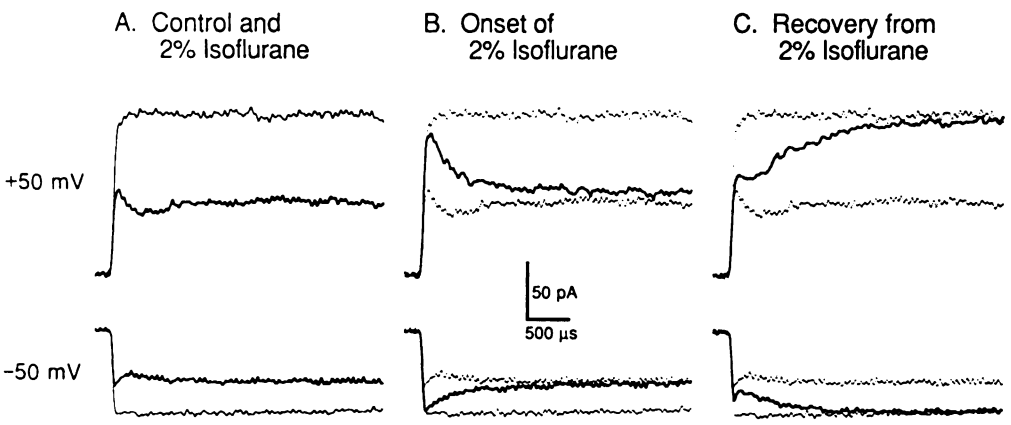


Fig. 6. High resolution current recordings from a fast patch during rapid application of 10 mM ACh at +50 mV (upper) and −50 mV (lower). Very little desensitization occurs during the 3.2 msec shown here. A, Current traces obtained under control (no isoflurane) and equilibrium (constant exposure to 2% isoflurane) conditions. The equilibrium traces have a smaller amplitude and are drawn as darker lines. Both control and equilibrium traces are redrawn in B and C as very light lines. B, The onset of inhibition by 2% isoflurane. The dark traces were obtained by having isoflurane present in the agonist-containing perfusion solution only. Thus, ACh and isoflurane were applied simultaneously. C, Recovery of inhibition by 2% isoflurane. The dark traces were obtained by having isoflurane present in the agonist-free perfusion solution only. Thus, isoflurane was removed from the patch as ACh was applied.

TABLE 1

Current decay time constants arising from rapid addition (onset) and removal (recovery) of 2% isoflurane

The results are shown as mean value ± standard deviation (number of determinations).

Voltage (mV)	Onset time constant (µsec)	Recovery time constant (µsec)
+50	300 ± 50 (10)	670 ± 130 (12)
−50	200 ± 100 (10)	480 ± 160 (13)

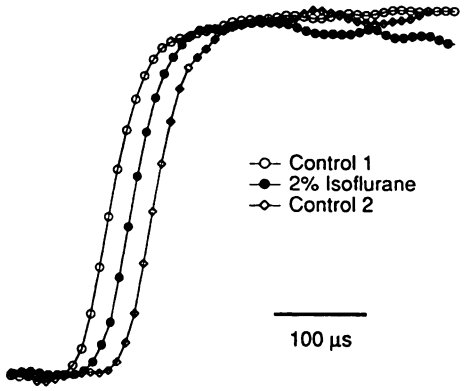


Fig. 7. A comparison of the onset of channel opening in control, during equilibrium application of 2% isoflurane and a second control after removal of isoflurane. The equilibrium current trace was scaled by a factor of 1.55 and displaced to the right by 20 µsec. The second control was displaced to the right by 40 µsec. The applied potential was +50 mV.

to promote new nonconducting conformations of the channel. Channel flickering arises as isoflurane (at a concentration [B]) repeatedly binds and blocks the channel (with the association rate constant f) and dissociates from the channel (rate constant b). A burst of channel activity ends when the gate of the channel closes in either the unblocked (α) or blocked conformation ($\alpha' > \alpha$).

This model is based on a simplification of the normal kinetic scheme for ACh receptor channels in which all closed states of the channel are represented by a single state (C). Although this simplification has some applicability to the activation of channels by low concentrations of ACh, it is woefully inadequate to describe the experiments in this paper. We must expand the model to account for (at the very least) three closed states corresponding to unliganded (R), singly liganded (AR), and doubly liganded (A_2R) closed states and one open state (A_2R^*) (8). Isoflurane can bind to any or all of these four states. The resulting scheme is shown as Scheme 2.

Here, the two ACh binding sites are considered to be identical and noncooperative (12, 13); the equilibrium binding constants are K_{eq} and K'_{eq}. Isoflurane may bind to and dissociate from each of the states with different rate constants; for simplicity, Scheme 2 explicitly shows only two sets of isoflurane binding constants. Whereas in Scheme 1, β and β' represent a combination of binding and isomerization rate constants, in Scheme 2, β and β' are the rates of channel opening (gating) in the absence and presence of isoflurane, respectively. For reasons presented previously (6), we do not consider the brief duration open state that occurs with a low frequency in these channels. The rate and equilibrium constants shown in Scheme 2 are not completely independent; they are constrained by microscopic reversibility.

Our experimental results can help assess the validity of Scheme 2 to describe the effects of isoflurane on ACh receptor channels and to determine values for the unknown parameters. First, consider the kinetics of inhibition by isoflurane illustrated in Fig. 6, B and C. Because 10 mM ACh was used in these experiments, the agonist binding steps in Scheme 2 are completed within 100 µsec and we need to consider only the four doubly liganded states. Moreover, because ACh is a very efficacious agonist (12), the occupancy of the open state (A_2R^*) is much greater than that of the closed state (A_2R). The scheme predicts that upon rapid application of isoflurane, the current decays as open channels become blocked ($A_2R^* \rightarrow A_2R^*B$). The time constant for this decay is given by $(f[B] + b)^{-1}$. Using the values of f and b obtained in our study of single channel kinetics (f = 1000/%/sec, b = 2000/sec, for −100 mV (6)), a time constant of 250 µsec is predicted. This agrees with our observed

values of the onset time constant (Table 1). Similarly, the time constant for recovery of current after removal of isoflurane is predicted to be 1/b = 500 μsec. This is also in agreement with our experimentally determined recovery time constants (Table 1). Scheme 2 predicts that, at equilibrium, isoflurane should block a fraction f[B]/(f[B]+b) of the current, or 50% at 2% isoflurane. This value lies within the range of our observed values (Figs. 2A and 6).

Next, consider the binding of isoflurane to closed channel states. The equilibrium current trace of Fig. 6A does not exhibit an appreciable decay. Such a decay would be expected if isoflurane had to re-equilibrate with the channel protein after the channels had opened. This suggests that the binding affinity of the channel for isoflurane does not depend on the conformation of the gate. In terms of Scheme 2, this means that $f'/b' \approx f/b$. If the small decay seen in Fig. 6A (but not in Fig. 7) is taken to be evidence of stronger binding of isoflurane to the open relative to the closed conformation of the channel, then f'/b' would be no smaller than $0.75 \times f/b$.

The dependence of the peak current induced by 100 μM ACh on isoflurane concentration can be derived from the four doubly liganded states in Scheme 2.

$$I_p = \left(1 + \frac{f[B]}{b}\right)^{-1} \tag{2}$$

The predictions of Eq. 2 with different values for the affinity of the isoflurane binding site (b/f), are shown with the data in Fig. 2A. A single value of affinity cannot be used to fit all of the data; the range of possible values is 1–3%. This includes the value of 2% that is predicted from our single channel kinetic measurements. The variability seen in Fig. 2A may arise from experimental difficulties (see Results) or from an inadequacy in Scheme 2. One possibility is that there are two isoflurane binding sites with somewhat different affinities.

We now turn our attention to the data obtained with non-saturating concentrations of ACh. Applying microscopic reversibility to the large cycle in Scheme 2 (omitting the $AR \rightarrow ARB$ and $A_2R \rightarrow A_2RB$ transitions), we obtain an expression for the open channel probability, the occupancy of the A_2R^* state.

$$p = \frac{c^2 \frac{\beta}{\alpha}}{1 + 2c + c^2\left(1 + \frac{\beta}{\alpha}\right) + \frac{f[B]}{b}\frac{K'^2_{eq}}{K^2_{eq}} \cdot \frac{\beta/\alpha}{\beta'/\alpha'}\left[1 + 2c' + c'^2\left(1 + \frac{\beta'}{\alpha'}\right)\right]} \tag{3}$$

In Eq. 3, $c = [ACh]/K_{eq}$ and $c' = [ACh]K'_{eq}$. For [B] = 0, Eq. 3 reduces to the expression for the open probability of the normal ACh receptor described by a four-state model (9). As [B] increases, the open probability at a given ACh concentration decreases. Thus, Scheme 2 predicts that, in the presence of isoflurane, the open probability cannot be larger than the open probability in control experiments. This is not in agreement with our observation that with rapid perfusion of 1 μM ACh, the peak open probability is 1.25 times greater in the presence of 1.9% isoflurane than in control (see Results). When we fit the remaining data in Fig. 5 to Eq. 3, we also encounter a problem. The data can be fit by assuming that either $K'_{eq} = 0.5 \times K_{eq}$ (ACh binds more tightly when isoflurane is bound) or $\beta'/\alpha' = 4 \times \beta/\alpha$ (channels open more quickly or close more slowly when isoflurane is bound). However, in each of these scenarios, microscopic reversibility demands that isoflurane binds about four times more tightly to open channels than to closed channels ($f'/b' = 0.25 \times f/b$). This contradicts our evidence that isoflurane binds to both conformations of the channel with nearly equal affinity (Fig. 6).

The previous paragraph suggests that the equilibrium values of both ACh binding and channel gating are the same in the upper and lower limbs of Scheme 2 ($K'_{eq} = K_{eq}$, $\beta'/\alpha' = \beta/\alpha$). Under these conditions, Eq. 3 simplifies to:

$$p = \left(1 + \frac{f[B]}{b}\right)^{-1} \frac{c^2 \frac{\beta}{\alpha}}{1 + 2c + c^2\left(1 + \frac{\beta}{\alpha}\right)} \tag{4}$$

Eq. 4 predicts that, in the presence of a constant concentration of isoflurane, the open probability is reduced by a constant factor at all concentrations of ACh. The data of Figs. 4 and 5 show that this is not the case; the apparent potency of isoflurane increases with increasing agonist concentrations. The dotted line in Fig. 5 is the prediction of Eq. 4 with b/f = 2.85% and the control values of K_{eq} = 56 μM, β/α = 17 (12). In order to explain our observations in terms of a model like Scheme 2 and the constraints imposed by the data, we must hypothesize another site of action for isoflurane. When isoflurane is bound to this additional site, either K_{eq} or β/α (or both) in *both* the upper and lower limbs of Scheme 2 is different from control. It is difficult to determine which of these parameters is changed by isoflurane, but there is some evidence that neither α nor β is affected by the anesthetic. Our measurement of the onset of channel gating by 10 mM ACh in a patch that does not exhibit an overshoot and undershoot (Fig. 7) suggests that the channel opening rate (β) is not affected by isoflurane. The concentration dependence of the channel open time (6) indicates that the closing rate (α) is not affected by isoflurane either. Our tentative conclusion is that the agonist affinity, K_{eq}, is a function of isoflurane concentration. The solid line in Fig. 5 is the prediction of Eq. 4 with K_{eq} = 40 μM. In this scenario, 1.9% isoflurane causes ACh to bind 1.4 times more tightly than normal. Eq. 4 predicts that at low concentrations of ACh (<3 μM), the open probability in the presence of 1.9% isoflurane should be about 15% greater than in control. This agrees with our observation that 1.9% isoflurane increased the current elicited with 1 μM ACh by 25% (see Results).

An increase in ACh binding affinity by isoflurane would also account for our observation that isoflurane increases the frequency of bursts seen in single channel recordings with 200 nM ACh (6). Since there are two agonist binding sites, the burst frequency is roughly proportional to K_{eq}^{-2} at low agonist concentrations.[2] We found that the burst frequency in the presence of 1.5% isoflurane was 1.8 times higher than in control (6).

Our study of the effects of isoflurane on desensitization is not comprehensive, but it does provide some useful information.

[2] There is no simple expression for the interval between bursts for Scheme 2, but analytical and numerical analyses show that, for the ACh receptor, the product of the agonist binding constants is a dominant factor (23).

In BC3H-1 cells, there is very little desensitization of ACh receptors in the absence of agonist (14, 18). In Fig. 6B, compare the equilibrium current with the onset current at the end of the traces: the current is the same whether or not the normal solution contained isoflurane. If isoflurane changes the degree of desensitization in the absence of agonist, the effect is too small to be detected in these experiments. The ACh receptors in Torpedo electric organ differ from those in BC3H-1 cells in that there is significant desensitization of unliganded receptors (18). Agonist binding studies suggest that volatile anesthetics increase the degree of desensitization in Torpedo ACh receptors in the absence of agonist (19).

In the normal ACh receptor in BC3H-1 cells, the rate-limiting step for recovery from desensitization is the transition from unliganded desensitized receptors to unliganded nondesensitized receptors (D→R) rather than the dissociation of agonist from desensitized receptors (14). The decrease in recovery rate in the presence of isoflurane (Fig. 3) may indicate either a decrease in the D→R transition rate or a decrease in the dissociation of agonist from desensitized receptors. Agonist binding studies with Torpedo ACh receptors indicate an increase in agonist binding affinity in the presence of volatile anesthetics (19, 20).

Isoflurane increases the rate of decay of current after application of agonist, but this increase is not concentration dependent (Fig. 2B). In our study of desensitization in BC3H-1 cells, we found that desensitization occurs primarily from the A_2R^* (open) state at high concentrations of ACh (14). Thus, the observed decay rate is equal to the product of occupancy of the open state and the rate constant for leaving this state. One possibility is that isoflurane increases the rate constant for desensitizing from the open state in a concentration-dependent fashion, but this is not manifested in the observed decay rate due to the tendency of isoflurane to decrease the occupancy of the open state.

The data presented here suggest that at least two different binding sites are needed to account for the effects of isoflurane on the ACh receptor channel. Isoflurane remains bound to one of these sites for relatively short times, on the order of 500 μsec. This site is responsible for the effects of isoflurane on flickering of single ACh receptor channels, on the transient current responses to rapid addition and rapid removal of isoflurane, and on the equilibrium current response to saturating agonist concentrations. We argued before that this site is likely to be somewhere on the ion channel protein itself, perhaps within the pore of the ion channel, or at the protein-lipid interface, rather than within the bulk lipid phase of the cell membrane (3, 6, 21). A strong piece of evidence for such a direct effect is our observation that the kinetics of onset and recovery of inhibition by isoflurane (Fig. 6, Table 1) correspond temporally with the frequency and duration of brief gaps (flickers) in single channel records.

An additional binding site (or sites) for isoflurane is needed to account for the effects of the anesthetic on agonist binding affinity and desensitization. When isoflurane is bound to this site, the binding of ACh to both the nondesensitized and desensitized conformations of the receptor is stabilized. Because agonist binding and dissociation occurs among electrically silent conformations of the channel, electrophysiological techniques give less direct information about these transitions. It is difficult to determine the time that isoflurane remains bound to this site and whether this site is more likely to be on the protein or in the surrounding lipid.

In this paper and the preceding one, we investigated the effects of isoflurane on several kinetic and equilibrium parameters of ACh receptor function. We have shown that the open/closed channel blocking model (Scheme 2) quantitatively accounts for some of these observations, in particular, mean channel open time and the peak current response to rapid perfusion of 100 μM ACh. However, the potency of isoflurane for reducing these two parameters by half is quite different: 0.4% for reduction of channel open time and 2% for inhibition of the peak current response. There is no contradiction in this. The model predicts different concentration dependencies for these parameters (compare Eq. 2 with $\tau_{open} = (\alpha + f[B])^{-1}$, Eq. 1 of Ref. 6). However, neither of these parameters directly determines the effectiveness of the ACh receptor at a synapse. The physiologically important factor is the amount of charge translocated across the postsynaptic membrane. Because the concentration of ACh at the synapse is essentially zero during the decay of the endplate current, the relevant quantity is the amount charge flowing through a single channel during a single burst (a burst ends when ACh dissociates from the receptor). The relative charge, q_{rel}, is equal to the product of the relative peak current amplitude (Eq. 2) and the relative burst duration (Eq. 5 of Ref. 6).

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

For the values of the rate constants determined from our single channel experiments (α = 310/sec, α' = 1100/sec, f = 1000/%/sec, b = 2000/sec, −100 mV, Ref.6), q_{rel} = 0.5 when [B] = 0.6% (300 μM at 25°)[3]. At this concentration, the peak amplitude is 77% of control and the burst duration is 66% of control. An earlier estimate of the EC_{50} for the inhibition of nicotinic ACh receptors by isoflurane is larger than this: 3.4% isoflurane (1) (800 μM at 37°). This estimate was based on measurements of the inhibition of agonist-induced muscle depolarization; these measurements are sensitive to the amplitude but not the time course of the depolarization and may also be affected by desensitization. Our results indicate that potency of isoflurane on neuromuscular transmission is similar to its potency as a general anesthetic (EC_{50} = 1.15% (22), 280 μM at 37°).

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