Gating kinetics of Shaker K⁺ channels are differentially modified by general anesthetics

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Correa, Ana M. Gating kinetics of Shaker K⁺ channels are differentially modified by general anesthetics. Am. J. Physiol. 275 (Cell Physiol. 44): C1009–C1021, 1998.—The Shaker B K⁺ channel was used as a model voltage-gated channel to probe the interaction of volatile general anesthetics with gating mechanisms. The effects of three anesthetics, chloroform (CHCl₃), isoflurane, and halothane, were studied using recombinant native and mutant Shaker channels expressed in Xenopus oocytes. Gating currents and macroscopic ionic currents were recorded with the cut-open oocyte voltage-clamp technique. The effects of CHCl₃ and isoflurane on gating kinetics of noninactivating mutants were opposite, whereas halothane had no effect. The effects on ionic currents were also agent dependent: CHCl₃ and halothane produced a reduction of the macroscopic conductance, whereas isoflurane increased it. The results indicate that the gating machinery of the channel is mostly insensitive to the anesthetics during activation until near the open state. The effects on the conductance are mainly due to changes in the transitions in and out of the open state. The data give support to direct protein-anesthetic interactions. The magnitude and nature of the effects invite reconsideration of Shaker-like K⁺ channels as important sites of action of general anesthetics.

Xenopus oocytes; chloroform; halothane; isoflurane; volatile anesthetics.
affect channel function by altering the kinetics of transitions near the open state.

MATERIALS AND METHODS

Materials

Frogs (Xenopus laevis) were purchased from either Xenopus 1 or Alcon. Halothane was from Halocarbon Laboratories, and isoflurane (Forane) was from Ohmeda Carbide. CHCl3 (chemical grade) and methane sulfonic acid (99%) were from Aldrich Chemical. All other chemicals were purchased from Sigma.

K+ Channel Clones and cRNA Transcription

The cDNA for the Drosophila ShakerB wild-type K+ channel was a generous gift of Dr. D. Papazian. All other clones used in this study were generously provided by Dr. L. Toro. They were the noninactivating mutant of the ShakerB channel, H4 IR, and the nonconducting mutant of the ShakerB channel, W434F, either with intact inactivation (W434F) or with inactivation removed (W434F IC). cDNAs for the various clones were transcribed with the commercial T7 mMessage mMACHINE kit from Ambion. The integrity of the transcribed RNA was assessed in agarose gels, and concentrations were determined spectrophotometrically.

Oocytes

Xenopus oocytes were isolated from frog ovaries, following conventional protocols described elsewhere (12). Oocytes were defolliculated by collagenase treatment (collagenase type AI, GIBCO BRL) in zero-Ca2+ Ringer, followed by a progressive change to the normal frog Ringer containing 1.8 mM Ca2+. Defolliculated oocytes were kept in Ringer solution (standard oocyte solution) that consisted of (in mM) 100 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.2. RNA samples (0.05–0.1 μg/μl) were injected into the oocytes within 1 day after isolation. The oocytes were tested for expression levels from 1 to 4 days after injection, depending on the K+ channel clone used. All animal procedures were approved by the Animal Research Council at the University of California, Los Angeles.

Recording Solutions

The external solution (in mM) was 120 potassium methane sulfonate, 1.8 CaCl2, and 10 HEPES (KMS ext; pH 7.2), 120 sodium methane sulfonate, 1.8 CaCl2, and 10 HEPES (NaMS ext; pH 7.2), or 60 sodium methane sulfonate, 60 potassium methane sulfonate, 1.8 CaCl2, and 10 HEPES (NaKMS ext; pH 7.2). The internal solution, and contact between the solution and the oocyte cytoplasm was established by permeabilizing the bottom of the oocyte with saponin (0.3%). Permeabilization was monitored by following the change in the capacitive transient for a 10-mV pulse. The oocyte was impaled with a low-resistance (<1 MΩ in 3 M KCl) glass electrode (VWR Scientific) at the center of the recording surface, and the electrode was kept close to the membrane for better voltage control. Low-resistance agar bridges, filled with 3% agar in 1 M sodium methane sulfonate, complemented by platinum wires threaded through, connected the chambers and the AgCl electrodes in 1 M NaCl.

Anesthetics

Solutions containing CHCl3 were prepared by dilution of a saturated (60 mM) solution in the external medium. Solutions containing the volatile anesthetics halothane and isoflurane were prepared by vaporizing the anesthetic in clinical vaporizers (Ohio) into external solution in an equilibrator (R. S. Weber and Associates, Westlake Village, CA) for 30 min. Equilibrated solutions were collected into gastight glass syringes. Solution exchange was done manually or via Teflon tubing in a gravity-fed perfusion system. Anesthetic concentration was determined by headspace sampling with a gas chromatograph (Hewlett-Packard HP 6890 Series with headspace sampler HP 7694) from an aliquot of the anesthetic-containing solution withdrawn directly from the equilibration chamber (halothane and isoflurane) or sealed vial (CHCl3), right before loading the perfusion syringes. The aliquot (200 μl) was rapidly placed at the bottom of a vial containing 5 ml of distilled water, and the vial was immediately sealed and kept for subsequent processing. The concentrations of the anesthetics used here are either higher than or equivalent to the minimum alveolar concentration (MAC) of each agent (MAC is 1 mM for CHCl3, 0.26 mM for halothane, and 0.4 mM for isoflurane). A clinically relevant concentration for CHCl3 is 1 mM (1 MAC). In some experiments, the saturated 60 mM CHCl3 solution was used. The highest concentrations of halothane and isoflurane used in this study correspond to 3.95 and 2.2 MAC, respectively. Clinically relevant concentration ranges are 0.5–2 MAC for halothane and 0.4–2 MAC for isoflurane.

Electrophysiology

Ionic currents and gating currents were recorded with the cut-open oocyte Vaseline gap voltage-clamp technique, as described elsewhere (24, 25), using a commercial voltage clamp (Clampator, DAGAN Instruments). Briefly, the oocyte was placed in a three-compartment chamber. Currents were recorded from an area of the oocyte of one-fifth to one-fourth of the total surface of the oocyte that was exposed in the top compartment. The middle compartment served as a guard for leakage currents between the bottom (internal medium) and the top (external medium) compartments. The solutions in the top and middle compartments were identical and were exchanged at the same time. The bottom chamber contained internal solution, and contact between the solution and the oocyte cytoplasm was established by permeabilizing the bottom of the oocyte with saponin (0.3%). Permeabilization was monitored by following the change in the capacitive transient for a 10-mV pulse. The oocyte was impaled with a low-resistance (<1 MΩ in 3 M KCl) glass electrode (VWR Scientific) at the center of the recording surface, and the electrode was kept close to the membrane for better voltage control. Low-resistance agar bridges, filled with 3% agar in 1 M sodium methane sulfonate, complemented by platinum wires threaded through, connected the chambers and the AgCl electrodes in 1 M NaCl.

A custom-built acquisition system (hardware and software) was used to elicit and record the data digitally. Data were acquired by sampling at 50–100 kHz for short pulses (10, 15, or 20 ms) and at 10–200 kHz for long pulses (45, 50, or 100 ms) and filtered at one-fifth of the sampling frequency. When required, the capacitive transient was subtracted online using P−/− protocols. Analysis was performed on the digitally stored data with Analysis, custom software written to complement the acquisition. Fits (single and double exponentials and Boltzmann distributions) were done with NFIT.

Fractional conductance data were obtained from the instantaneous value of the ionic currents upon repolarization. In practice, the peak tail current was measured isochronally at a point close to the repolarization time (see Fig. 5C; measurement made at 0.62 ms for the conductance-voltage (G-V) curve) or from the fit of the tail currents to exponential functions extrapolated to time 0 (see G-V curves in Figs. 2D and 7C). The latter method was normally used to analyze G-V data in experiments in which the anesthetic caused a change in kinetics. G-V curves were fitted to sequential models with Boltzmann distributions based on the state sequence C0,C1,C2,O,Ci, where the Ci (i = 0, 1, 2) are closed states leading to an open state (O). The blocked state (Ci) is populated from the open state. Ci was proposed by Hoshi et al.
(15) to account for a fast flickery state observed at depolarized potentials in single-channel data analysis of the IR mutant of the ShakerB channel. The single-channel data were analyzed with consideration of various possibilities for the location of this C2 state; the best fit to the data was given by models in which this state is accessed from the open state. The macroscopic manifestation of this state is a reduced probability of being open and a small 10% decline in the peak current after a few milliseconds. The extent of occupancy of the O \( \approx C \) transition varies among oocytes (23). In the inactivating channel, inactivated state(s) would stem in parallel from the open state (not shown) (15). Also, the first two transitions carry most of the gating charge, whereas C2 \( \approx O \) and O \( \approx C \) are only slightly voltage dependent, with only \(-0.4 \) charges and \(< 0.1 \) charges, respectively (23). The equation used to fit the G-V data was of the form

\[
G = G_{\text{max}} B \cdot \exp(-z_1 V/25) / [1 + B \cdot \exp(-z_3 V/25)] \cdot [1 + A \cdot \exp(-z_2 (V - V_i)/25)] \cdot [1 + \exp(-z_0 (V - V_o)/25)]
\]

A simplified version, based on the sequence \( C_0 \approx C_1 \approx O \), was also used

\[
G = G_{\text{max}}/[1 + \exp(-z_2 (V - V_i)/25)] \cdot [1 + \exp(-z_0 (V - V_o)/25)]
\]

In both, \( G_{\text{max}} \) is the maximal conductance, \( z_i \) are the valences \( i = 0, 1, 2, 3 \), and \( V_i \) are the midpoint potentials of the transitions \( i = 0, 1, 2 \). A and B represent the ratios of the backward (\( b_i \)) to the forward (\( a_i \)) transition rates at zero voltage between \( C_2 \approx O \) and \( O \approx C_1 \). These data were first fitted letting all parameters be free. For all G-V data sets, the values of \( z_i \) did not change significantly between control and anesthetic treatment data. This indicated that the anesthetics did not affect the valence of the transitions. Therefore, the best values for \( z_i \) were determined by iterating between control and experimental data. Once appropriate \( z_i \) had been found, i.e., both data sets could be fitted well with the same values, these were held constant while the best fits for \( G_{\text{max}}, V_i, A, \) and B were obtained. The following constraints were applied to maintain consistency with previous studies of this same channel (15, 23); all valences were positive; the transitions \( C_2 \approx O \) and \( O \approx C_1 \) are only mildly voltage dependent, and therefore upper limits were set for \( z_2 \) at 0.5 and for \( z_3 \) at 0.2; and A was constrained to \(< 1 \) in the control to account for the normal opening probability of the channel. In the final fit of the control and experimental G-V data, \( G_{\text{max}} \) and either \( V_o \) for CHCl3 or \( V_i \) for isoflurane were maintained at fixed values, which effectively reduced the number of parameters to fit. A good fit of the experimental data could be achieved by keeping the control values for these parameters in the fit of the CHCl3 and isoflurane G-V data. On the other hand, when A, B, and either \( V_i \) (CHCl3) or \( V_o \) (isoflurane) were set to the control values, the experimental data could not be fitted accurately, thus these parameters were clearly changing in the presence of the anesthetics. All parameters in Table 1 were obtained as described above.

### RESULTS

To address the question of whether volatile anesthetics interact directly with the protein of a voltage-gated channel, the nature and specificity of the effects on the gating mechanisms of the Shaker \( K^+ \) channel were studied by recording gating currents from oocytes expressing the nonconducting (W434F), noninactivating (IR) mutant. Gating charge movement on the order of 1 \( \mu \)C can be recorded from oocytes 1 day after injection (e.g., see Ref. 22). The effect of the anesthetics on the gating charge was evaluated by studying the anesthetic-induced changes in the magnitude and kinetics of the charge movement and in their voltage dependence.

Experiments in CHCl3. Noninactivating ShakerB channel. GATING CURRENTS. Figure 1 shows the results obtained by exposing W434F IR channels to 1 and 60 mM CHCl3. In Fig. 1A, superimposed control and experimental gating currents records. CHCl3 (1 mM) had little or no effect on either the magnitude or the time course of the "on" gating charge movement (\( I_{g,\text{on}} \)), whereas it considerably accelerated the return of the gating charge (\( I_{g,\text{off}} \)). The CHCl3-induced faster relaxation of \( I_{g,\text{off}} \) was absent at \(-60 \) mV, developed at potentials more positive than \(-50 \) mV, and became clear at the more depolarized potentials. The acceleration of \( I_{g,\text{off}} \) produced by 60 mM

### Table 1. Fits of G-V curves in Figs. 2D, 5C, and 7C to sequential Boltzmann distributions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Experimental</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>( G_{\text{max}} ) (1 mM)</td>
<td>67.21</td>
<td>67.21</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( B )</td>
<td>2.89</td>
<td>2.41</td>
<td>( \uparrow )</td>
</tr>
<tr>
<td>( z_i )</td>
<td>0.05</td>
<td>0.05</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( A )</td>
<td>0.05</td>
<td>0.16</td>
<td>( \uparrow )</td>
</tr>
<tr>
<td>( z_i )</td>
<td>0.43</td>
<td>0.43</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( V_0 )</td>
<td>2.16</td>
<td>2.16</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( V_1 )</td>
<td>-3.96</td>
<td>-15.59</td>
<td>( \uparrow )</td>
</tr>
<tr>
<td>( z_0 )</td>
<td>4.97</td>
<td>4.97</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( V_1 )</td>
<td>-26.70</td>
<td>-26.70</td>
<td>( \downarrow )</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>1 \times 10^{-1}</td>
<td>6.3 \times 10^{-2}</td>
<td>( \downarrow )</td>
</tr>
</tbody>
</table>

Data for chloroform and isoflurane were fitted to Eq. 1 (see text); \( A = \exp(\pm V_2 V_3) \) and \( B = \exp(\pm V_2 V_4) \). \( G_{\text{max}} \), maximal conductance; \( z_i \), slope; \( V_i \), midpoint potential. Data for halothane were fitted to Eq. 2. *Values of indicated parameters were held constant during fitting of data (see MATERIALS AND METHODS). Double arrows indicate 3-fold or larger change over single arrows.
Fig. 1. CHCl₃ modifies gating charge movement of nonconducting, noninactivating W434F IR Shaker K⁺ channel.

A: CHCl₃ accelerates return of gating charge upon repolarization without a significant parallel effect on kinetics of gating charge movement triggered by depolarization. Superimposed records of gating charge movement obtained by stepping membrane from holding potential (Vₜₜ) of −80 mV to potentials indicated. Data for control (thin lines) and CHCl₃ (thick lines) were recorded in external solution of NaKMSₜₑₓₜ and internal solution of KMSᵢⁿ. (see Recording Solutions). CHCl₃ data were recorded immediately after exchange of control external solution with solution containing 1 mM CHCl₃.

B: CHCl₃-induced changes in kinetics of Iᵩᵩ are voltage dependent and coincide with channel opening. Time constants of decay (τ) of on and off gating currents are plotted as function of test depolarization during 40-ms pulses to between −50 and +60 mV from Vₜₜ, and with return to −90 mV. Time course of decay of Iᵩᵩ (circles) and Iᵩᵩ (triangles) was fitted assuming single-exponential relaxation (τ are on log₁₀ scale). Filled and open symbols, control and 60 mM CHCl₃, respectively. Recordings were done in external NaMSₜₑₓₜ and internal KGᵢⁿ. Bars, SD for potentials at which 2 measures were made. Inset: superimposed traces of gating currents recorded with pulse to 0 mV from Vₜₜ of −90 mV. Scale bars, 10 ms and 500 nA. Arrow, 60 mM CHCl₃ trace.

C: channel closing is accelerated by CHCl₃ irrespective of potential of repolarization. Tail current protocols were used to determine time course of Iᵩᵩ after 40-ms step depolarization to +20 mV, when open probability is high. Membrane was then stepped back to potentials between −150 and −40 mV. Full time course of Iᵩᵩ at each tail potential was fitted to double-exponential function. Resulting control (filled symbols) and experimental (open symbols) fast (τᵩᵩ; circles) and slow (τᵩᵩ; triangles) time constants are plotted as function of tail potential. CHCl₃ (60 mM) induced reduction of τᵩᵩ (rising phase) and τᵩᵩ (falling phase) at all potentials tested. Same recording conditions as B. D: absolute values of amplitudes of exponential functions described in C are plotted as function of tail potential. CHCl₃ induced increases in fast and slow components. Symbols as in C. E: CHCl₃ did not affect voltage dependence of charge movement. Charge-voltage (Q₋₋₋₋₋₋) plot shows integrated charge moved upon depolarization (Q₋₋₋₋₋) and repolarization (Q₋₋₋₋₋₋) plotted vs. pulse potential (ncoul, nanocoulombs). Control and CHCl₃ data superimpose, suggesting no significant effect on voltage dependence of charge movement. Also, no excess gating charge was observed in CHCl₃, showing that anesthetic accelerates movement of charge and does not contribute additional charge.
CHCl₃ was larger, as illustrated with the superimposed traces shown in Fig. 1B, inset. As at the lower concentration of CHCl₃, the acceleration of the return of the gating charge develops with depolarization becoming evident at potentials at which the channels open (e.g., see G-V relation in Fig. 2D). This is clearly seen in the plot shown in Fig. 1B. The time constants (τ) of the decay phases of I_{g,on} and I_{g,off} revealed that control and treated on and off gating currents had similar time courses at potentials at which the probability of opening is low, below −30 mV. At the potentials at which the channels are open, I_{g,off} in CHCl₃ (60 mM) is fivefold faster than the control (5.1 ± 0.3, mean ± SE, from −20 to +60 mV). I_{g,on}, on the other hand, decayed with similar kinetics in control and experimental data. There was an average 5 ± 2% reduction in the τ of decay of I_{g,on} between −10 and 60 mV. Acceleration of I_{g,off} was seen in 9 of 11 cells studied with 60, 10, or 1 mM CHCl₃, the effects were reversible in 8 of the 9 cells and ranged from 1.5- to 9-fold reduction in the τ.

The effect of CHCl₃ on I_{g,off} was further investigated in tail current experiments (Fig. 1, C and D). CHCl₃ accelerated the rate of rise and of decay of I_{g,off}. Where the reduction in the time constant of the faster exponential (τ₁) was nearly constant (73 ± 1\% in the range −120 to −70 mV, the change in the time constant of the slower exponential (τ₂) increased steadily from 30% reduction at −120 mV to 77% at −70 mV (Fig. 1C). The voltage dependence of the rise of I_{g,off} (τ₁) was unaffected by the CHCl₃, τ₂ in CHCl₃ was less dependent on potential than the control. The amplitudes of rising and falling phases of I_{g,off} (Fig. 1D) were increased proportionately by CHCl₃.

In addition, CHCl₃ did not affect the voltage dependence of the total gating charge moved (Fig. 1E). The superposition of the charge-voltage (Q-V) curves shown in Fig. 1E indicates that in CHCl₃ there was no appreciable shift or change in the slope of the Q-V relation. Because the W434F IR is a nonactivating channel, the charge moved with depolarization (Q_{on}) returns upon repolarization, i.e., Q_{on} should equal the charge moved with repolarization (Q_{off}) during the normal integration period. Q_{on} and Q_{off} in control and in CHCl₃ overlap, suggesting that the charge is recovered in the anesthetic-treated channels and that no additional charge was contributed by I_{g,off} when in CHCl₃. The effects were fully reversible after washout. The results indicated that the effects of CHCl₃ were occurring in the vicinity of the open state, since they became evident at potentials at which the channels are expected to be open. This prompted me to look at the properties of the conducting channel and, also, those of inactivation that modifies the open state of the channel.

**Ionic currents.** To look at the consequence of CHCl₃ treatment on the ionic conductance, K⁺ currents were recorded from oocytes expressing the ShakerB conducting H4 IR mutant (wild-type Shaker channel with reduced inactivation). The effects of CHCl₃ on the nonactivating H4 1R were consistent with the data obtained in gating current experiments with the H4 W434F 1R mutant mentioned above. CHCl₃ did not alter the kinetics of the ionic currents of H4 IR channels in response to test depolarizations, i.e., currents could be scaled to match the control records, but it increased the rate of deactivation of the ionic currents. This is illustrated by the family of curves shown in Fig. 2A, the superimposed traces in Fig. 2B, and the plot in Fig. 2C. CHCl₃ (1 mM) produced a reversible inhibition of 12–14% that was constant for potentials ranging from −10 to +60 mV. There was no change in the reversal potential, indicating no change in ionic selectivity or in the leak. At higher concentrations, CHCl₃ (8 mM) induced an important inhibition of the ionic currents (e.g., currents were reduced to 10% at +60 mV) that was partially reversed (50%) with washout (data not shown). Block of the ionic currents by CHCl₃ was observed in five of six oocytes tested. At low (1 mM) and high (8 and 60 mM) concentrations of CHCl₃, the block of the ionic tail currents was larger than that of the currents during the pulse: 22.3 ± 6.6 vs. 17.3 ± 6.0\% and 94.4 ± 2.5 vs. 84.4 ± 3.8\%, respectively.

The time course of the tail currents was accelerated by the CHCl₃, as expected from the acceleration of the off gating charge in the W434F IR mutant. This is illustrated by the superimposed control, CHCl₃, and normalized CHCl₃ traces shown in Fig. 2B. The analysis of the rate of decay of tail currents (Fig. 2C) indicated that CHCl₃ produced a 30% decrease in τ_{g,off}, which is the main component describing the tails. It also induced a substantial reduction in τ_{g,off}, e.g., 70\% at +5 mV (Fig. 2C, inset). CHCl₃ produced a >30% reduction of the amplitude of the main, fast component of the tail current, without a parallel effect on the slow component (33 ± 6 vs. 0.3 ± 9\%); in control and CHCl₃ data, the fast component was larger than the slow component by 24 ± 2- and 39 ± 5-fold, respectively (data not shown). The change in time constants was concomitant with channel opening, which correlates well with the change seen in the gating current experiments. These results suggested that the decrease in the macroscopic conductance could be brought about by an increased rate of deactivation (channel closing).

The results obtained from the analysis of the G-V relations are consistent with the idea that CHCl₃ reduces the maximum conductance by destabilizing the open state and by causing a hyperpolarizing shift in the voltage dependence of the conductance. In Fig. 2D we plotted the G-V curves obtained from the tail currents repolarizing back to −50 mV. CHCl₃ (1 mM) induced a depression of the G-V at potentials more positive than −30 mV. The solid lines are the fits of the data to a five-state sequential model, as described in MATERIALS AND METHODS. The parameters for the fit [G_{max}, B, A, E (i = 0, 1, 2, 3), V_{r}, and V_{z}] are shown in Table 1. Control and experimental data were well fitted by adjusting only B, A, and V_{z}. The anesthetic produced a 12-mV hyperpolarizing shift in V_{r} (V_{r} = −4 mV for control and −16 mV for CHCl₃), an increase in the value of A from 0.05 to 0.16, and a reduction in B from 2.89 to 2.41. The progressive reduction in conductance at very depolarized potentials is accounted for by incorporating the
transition $O \rightarrow C_f$ (see MATERIALS AND METHODS). An increase in parameter $A$ was expected from the time course of the ionic tails and of $I_{g,off}$ because it is proportional to $b_2/a_2$, the ratio of the rates out of and into the open state $O$. Thus an increase in $b$, the rate at which the channel closes, would produce an increase in $A$. Likewise, the parameter $B$ is proportional to $b_3/a_3$; a reduction in $B$ implies an increase in $a_3$ or a decrease in $b_3$ and, in both cases, the open state would be less populated. These two changes could account for a reduction in the ionic conductance. They were, however, insufficient to account for all the changes produced by the CHCl$_3$. A change in $V_1$ was required for proper fit of the data, indicating that CHCl$_3$ had a dual effect: it destabilized the open state and produced a leftward shift in the G-V. A shift in the voltage dependence of transitions leading to the open state was not obvious from the Q-V data obtained with the W434F mutant; this may be because the transition involved in the shift carries only a fraction of the charge, 30%, which could escape detection with the scatter in the Q-V data. Clearly, the rates of transitions in and out of the open state were being affected by the CHCl$_3$, and therefore the process of inactivation could also be affected by the anesthetic. This was investigated by recording gating and ionic currents from nonconducting (W434F) and conducting inactivating Shaker channels.

Inactivating ShakerB channel. GATING CURRENTS. In contrast to the results with the W434F 1R mutant (Fig. 1), the effect of CHCl$_3$ on the kinetics of $I_{g,off}$ was prevented in channels with intact inactivation. This is
shown in Fig. 3, inset. It is clear that in the inactivating W434F channel, \( I_{\text{g,off}} \) were mostly immobilized and had similar kinetics in CHCl\(_3\) and in the control. \( I_{\text{g,on}} \), on the other hand, appeared to be slightly accelerated in CHCl\(_3\). The \( Q-V \) plot shown in Fig. 3 illustrates that the total charge moved (\( Q_{\text{on}}, Q_{\text{off}} \)) at each potential remained the same in CHCl\(_3\). There is also no evidence of a change in the voltage dependence of the gating charge movement following CHCl\(_3\) treatment. \( Q_{\text{off}} \) was immobilized in CHCl\(_3\) to the same extent and with the same voltage dependence as the control, indicating that the anesthetic-induced acceleration of \( I_{\text{g,off}} \) seen in the absence of inactivation is prevented or not detectable for all voltages when inactivation takes place.

**IONIC CURRENTS.** Examination of the ionic currents in the wild-type inactivating channel confirmed the lack of effect of CHCl\(_3\) when inactivation is intact. CHCl\(_3\) (1 mM) had a small effect on the ionic currents of the wild-type ShakerB channel (data not shown). The kinetics of activation and inactivation were slower in CHCl\(_3\). Regardless of the length of the depolarizing epoch (e.g., 3 or 15 ms), tail current kinetics were mostly unaffected, as was the \( G-V \) relation. This indicates that the effect of 1 mM CHCl\(_3\) is not state dependent but rather is dependent on the presence or absence of the inactivating particle. The differential effects of CHCl\(_3\), dependent on the integrity of the process of inactivation, suggest that the effects require a direct interaction between the anesthetic and the channel protein. Also, all results taken together would predict that native A-type currents would be minimally affected by CHCl\(_3\), whereas the noninactivating variants of Shaker-like channels would tend to conduct less and close faster as the membrane repolarizes.

**Experiments in Halothane**

Gating currents. Halothane had no effect on the kinetics or magnitude of the gating charge movement of the W434F IR channel, as illustrated in Fig. 4A. The traces at the top are currents recorded with 1 mM CHCl\(_3\). After washout of CHCl\(_3\), the oocyte was subjected to 2.5 mM halothane. The H4 W434F IR channel...
clearly responded to the CHCl₃ but not to the halothane applied immediately afterward. Halothane had no effect on gating currents in four oocytes tested. In two of these, CHCl₃ produced acceleration of Iₒ.off. This same high concentration of halothane was used in an oocyte expressing wild-type H4 W434F channels (Fig. 4B). As in the case of the W434F IR (Fig. 4A), even at this high concentration, halothane had only minimal effects on the kinetics of the gating charge. The Q-V curves for Qₒ, and Qₒ.off in control and halothane-treated channels superimposed (data not shown). Ionic currents through the inactivating and noninactivating conducting channels were expected to be unaffected by halothane.

Ionic currents. Halothane had no effect on channel kinetics (Fig. 5). There was no effect on the rates of activation or deactivation of H4 IR channels, as evidenced by the lack of effect on the time course of the macroscopic ionic currents (Fig. 5A). In the presence of a relatively high concentration of halothane (2.5 mM for the data shown in Fig. 5), ionic currents were depressed by 10–20% [12% in the current-voltage (I-V) plots in Fig. 5B]. There was a block of the conductance (28% in the G-V in Fig. 5C and Table 1), which became constant for voltages positive to −10 mV. The fractional conductance curves of control and halothane-treated currents superimposed (Fig. 5C, inset), indicating no change in the voltage dependence of the conductance. This result differs from that with CHCl₃ in that halothane, as expected from the gating current experiments, did not produce changes in the kinetics of the ionic conductance, suggesting that it may mostly affect the single-channel conductance. The blocking effect of halothane on the ionic currents was observed in five of five oocytes; the average block of 14.8 ± 2.8% was reversible after washout.

Experiments in Isoflurane

Gating currents. Isoflurane had a marked effect on the kinetics of the gating charge movement in W434F IR channels. The most dramatic effect was a considerable slowing down of Iₒ.off, as shown in the superimposed traces in Fig. 6A. Again, the effect was more pronounced at higher depolarizations. The time constants of the decay of Iₒ.on and Iₒ.off are shown in Fig. 6B, top and bottom, respectively. Isoflurane induced a small increase in both γₒ and τₒ values for Iₒ.on. In contrast, isoflurane doubled the time constant of the off gating currents for pulses more positive than −40 mV. At potentials at which the probability of opening is small, isoflurane had only a small effect. This was observed in two of three oocytes tested; the effect was reversible and amounted to 2.4- and 3.4-fold increases in the time constant of decay of Iₒ.off. The effect of isoflurane, although opposite, was similar to that of CHCl₃ and halothane in that it became evident as channels opened and in that there was no effect on the voltage dependence of the gating process (there was only a small depolarizing shift in the Qₒ, vs. V relation; not shown). The consequence of the kinetic change induced by isoflurane near the open state of the channel on channel conductance was explored in the conducting H4 IR channel.

Ionic currents. Currents through the noninactivating mutant H4 IR (Fig. 7) were dramatically affected by isoflurane. The anesthetic induced an increase in the current elicited by test depolarizations (I-V plot in Fig. 7B).
7A and Fig. 7A, inset); in the example, the increase was 30%. Isoflurane clearly caused a decreased rate of deactivation, as the tail currents appear to be larger and more prolonged (Fig. 7A, inset). The change in the time course of the tail currents induced by isoflurane is illustrated in the semi-logarithmic plot in Fig. 7B. The time constants of both fast and slow components are increased in isoflurane compared with the control and the wash. Isoflurane produced an increase in the time constants (>4-fold for $\tau_f$ at $-50$ mV) that is consistent with the reduction in the rate of return of the gating charge in the nonconducting H4 W434F IR mutant. A pronounced increase in the conductance was obtained from the G-V relations (Fig. 7C); it was higher at the foot of the G-V curve (e.g., 6-fold at $-30$ mV) than at more depolarized potentials (e.g., 1.5-fold at $+60$ mV). This is also evident from the fractional conductance curves (Fig. 7C, inset), from which it is apparent that the conductance started developing at more negative potentials in isoflurane than in the control. This was quantified further by fitting the data to the five-state model, $C_0 \rightleftharpoons C_1 \rightleftharpoons C_2 \rightleftharpoons O \rightleftharpoons C_r$, described in MATERIALS AND METHODS (Fig. 7C). In isoflurane there is a leftward shift in the voltage of activation of the channels (3 mV for $V_1$), a fourfold decrease in $A$, and a strong eightfold increase in $B$ (Table 1). The change in $A$ was expected from the slower return of $I_{g,off}$ and tail currents when in isoflurane. The change in $B$ indicates that it is much less likely to find the channel in the blocked state. Both contribute to an increase in the conductance (see Molecular Mechanisms below). Isoflurane induced an increase in the ionic current in 9 of 12 oocytes (ratio of isoflurane to control: peak ionic current, $1.35 \pm 0.15$; peak tail current, $1.45 \pm 0.06$) and slowed down tail current kinetics in 11 of 12 cells (ratio of isoflurane to control: $\tau_f$, $1.5 \pm 0.1$; $\tau_s$, $2.9 \pm 1.1$); the effects were reversible after washout in 9 of the 12 cells.

**DISCUSSION**

The ShakerB K⁺ channel was used as a model voltage-dependent ion channel to study the effects of three volatile anesthetics on its gating properties and on the voltage-dependent characteristics of the ionic conductance. The results can be summarized as follows. 1) The volatile anesthetics differentially affected the kinetics of the return of the gating charge in the nonactivating channel: CHCl₃ accelerated it, halothane had no effect, and isoflurane slowed it considerably. 2) The effect on $I_{g,off}$ was small, and there was no effect in the total amount of charge moved. 3) In channels with intact inactivation, CHCl₃ and halothane had little effect on the magnitude or kinetics of the gating charge movement. 4) Halothane and CHCl₃ produced a block of the ionic conductance, whereas isoflurane increased it. 5) Analysis of the G-V data indicated that none of the anesthetics produced changes in the voltage dependence of the conductances (there were no changes in $z$). 6) CHCl₃ induced a hyperpolarizing shift in the G-V and a destabilization of the open...
state by increasing the rates of exit from the open state to the last closed state and the blocked state. Halothane produced a small depolarizing shift and a reduction of the maximal conductance. Isoflurane produced a small hyperpolarizing shift and a stabilization of the open state; in isoflurane, there was strong destabilization of the blocked state.

The effects of the three anesthetics were seen as channels opened.

Direct Effects on the Channel Protein

The data presented in this paper suggest that inhalation anesthetics interact directly with the channel protein of voltage-gated Shaker $\mathrm{K}^+$ channels and not through an indirect effect on bilayer properties. The effect is selective in that the three anesthetics tested had different effects on the function of the channels. The data suggest that the gating machinery involved in channel activation does not respond to the presence of the anesthetics until transitions close to channel opening. If it is assumed that the anesthetics reach and change the environment of hydrophobic regions within the protein, the small effects on $I_{\text{g, on}}$ imply that the main charge-contributing transitions occur independently of changes in the hydrophobic environment close to where these gating charges are moving. These data do not rule out, however, possible effects on uncharged steps or on transitions carrying less charge and moving at early stages in the activation pathway. Effects of the anesthetics on such steps would have been detected had they introduced a rate-limiting step at earlier stages in channel activation. Although the effects of $\mathrm{CHCl}_3$, halothane, and isoflurane on the kinetics of $I_{\text{g, on}}$ of the Shaker channels were small, the effects on the return of the gating charge and channel closing were considerable and were very much dependent on the anesthetic. Although the nature of the effects, however small, on $I_{\text{g, on}}$ could be related to anesthetic-induced changes in the properties of the bilayer, it is hard to envision a common mechanism of action mediated by anesthetic effects on bilayer properties to explain the differential effect of $\mathrm{CHCl}_3$, halothane, and isoflurane on $I_{\text{g, off}}$ and tail kinetics.

The effects of $\mathrm{CHCl}_3$ on $I_{\text{g, on}}$ of the Shaker channels differ from those previously reported for the $\mathrm{Na}^+$ channel of the squid giant axon by Fernandez et al. (10). They observed that $\mathrm{CHCl}_3$, at high concentrations (60 mM), produced a block of the fast component of $I_{\text{g, on}}$ with a concomitant full block of the $\mathrm{Na}^+$ conductance. The slow component of the $I_{\text{g, on}}$ was not affected by the $\mathrm{CHCl}_3$. In contrast, the kinetics of the movement of dipicrylamine (a lipophilic ion) were dramatically accelerated by the same concentration of the anesthetic. The results were interpreted to imply that the effect of the anesthetic on the gating charge movement was not merely an effect of the physical properties of the bilayer but a direct effect on the gating machinery of the channel. With 60 mM $\mathrm{CHCl}_3$, the conductance of the Shaker channel was also fully blocked (data not shown), but this occurred without a block of any of the components of $I_{\text{g, on}}$ and with a small, but variable, change in

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**Fig. 7.** Iso increases macroscopic ionic conductance of Shaker H4 IR channel. A: I-V plots are current magnitudes at end of 25-ms depolarization vs. test potential. Currents recorded in external NaKMSext and internal KMSint. Inset: superimposed traces show increase of $\mathrm{K}^+$ current during pulse and after repolarization produced by 1.8 mM Iso. Pulse to +10 mV from $V_h$ of -70 mV. B: $t_\text{f}$ and $t_\text{s}$ describing decay of tail currents are plotted as function of tail potential. In experiment, tails were recorded at voltages from -120 mV to -50 mV after 15-ms depolarizing pulse to -50 mV. Iso produced an increase in $t_\text{f}$ and $t_\text{s}$ at all potentials that was reversible after washout. C: G-V curves from tail currents before (○) and after (●) treatment with 1.8 mM Iso. Solid lines are fits to 5-state sequential model (Eq. 1) as in Fig. 2D, keeping all $z_i$, $G_{\text{max}}$, and $V_1$ fixed. Fitted parameters $A$, $B$, and $V_2$ are in Table 1. Inset: fractional conductance curves show shift produced by Iso toward more hyperpolarized potentials.
kinetics. The difference in the effects of CHCl3 on the two channels (Na+ and K+) gives further support to specific interactions of the anesthetics with the channel protein.

Molecular Mechanisms

The results indicate a clear qualitative difference in the modulation of K+ channels produced by the different anesthetics. To gain more insight into the mechanisms by which the anesthetics may be affecting the noninactivating channels, we have modeled the effects of the anesthetics on the G-V relations of the conducting channels. Several models for the gating of ShakerB K+ channels have been proposed (2, 15, 23). The simplified version of one such model, after Rodriguez and Bezanilla (23), used in this study is shown in the scheme

\[
C_0 \xrightarrow{\alpha_0} C_1 \xrightarrow{\alpha_1} C_2 \xrightarrow{\alpha_2} O \xrightarrow{\alpha_3} C_f
\]

\[
\beta_0 \quad \beta_1 \quad \beta_2 \quad \beta_3
\]

As mentioned above, it is the transitions around the open state of the channel that seem to be modified by the anesthetics. When challenged with CHCl3 and halothane, the channels exhibited a reduced conductance. In the case of CHCl3, the change in the conductance seems to be brought about by accelerated rates of exit from the open state. The hyperpolarizing shift in the fitted V1, which is associated with the transition C1 \xrightarrow{\alpha_1} C_2, compensated partially for the change in A, the equilibrium between C2 \xrightarrow{\alpha_2} O. The increase of parameter A with CHCl3 was expected, since ionic and gating currents exhibited a faster return. The decrease in B indicates that either the channels visit the blocked state more with the anesthetic or that the transit from C1 back to O is reduced. Both changes would produce an apparent decrease in the conductance. With isoflurane, the main effect on the conductance was due to a reduction in the rate of deactivation and in the occupancy of the blocked state. The decrease in A with isoflurane was expected from the slower kinetics of I_{off} and tail currents. The change in B indicates that it is much less likely to find the channel in the blocked state; either the access (O to C1) is limited or the return (C1 to O) is much enhanced by the anesthetic. Both would increase the dwell time in the open state and therefore would produce an apparent increase in the conductance. Because the total on gating charge was not affected by any of the anesthetics, the changes in the conductance must occur without a change in the total number of channels. This again is consistent with the model results in that the G_{max} did not change in CHCl3 or in isoflurane and all changes in conductance arose from a modified dwell time in the open state.

With halothane, there was no clear evidence of kinetic changes, therefore, most of the effect was expected to be in the conductance proper. Indeed the model predicted a reduced G_{max} for halothane. This could be the result of single-channel conductance reduction or an entry to a blocked state.

The inactivation particle precluded the effect of CHCl3 on the return of the gating charge and on channel deactivation. This observation suggests that the site with which CHCl3 interacts is not exposed to the anesthetic when the inactivating particle is tethered to the channel. Alternatively, the binding of the inactivation particle to its docking site does not allow the CHCl3 effect to reach completion. Because hydrophobic interactions play an important role in the docking of the inactivation particle at its site (20), it is tempting to speculate that CHCl3 could interact with that site to produce a change that eases the return of the gating charge upon repolarization but with a much decreased affinity than the inactivating particle itself. The data on the wild-type Shaker channel suggest, however, that the influence of the inactivating particle on the action of 1 mM CHCl3 is not state dependent, because the anesthetic does not affect the closing rate of the fraction of channels that have not inactivated even at short depolarizations (data not shown).

Relevance to K+ Channel Function in General Anesthesia

K+ currents contribute to the control of neuronal excitability and are, therefore, relevant to general anesthesia. For example, A-type currents play a critical role in determining the output of CA1 pyramidal neurons by dampening dendritic excitability (14). Propagation of the action potential is controlled by an A-type conductance in hippocampus (5). Also, K+ currents have been implicated in the development of anesthetic sensitivity in Drosophila (27). In those studies, impairment of A-type currents resulted in behavioral insensitivity to isoflurane. It is important, therefore, to understand which channels are involved in the response to general anesthetics and what are the molecular mechanisms responsible for their action.

During an action potential, the changes in membrane potential drive the opening and closing of channels as determined by the voltage dependence and rates of the gating processes. Delayed rectifier K+ currents are important for membrane repolarization during an action potential, and the resting K+ conductance determines the membrane potential and, consequently, excitability. The H4 IR mutant studied here has characteristics of a delayed rectifier channel. An acceleration of channel closing alongside a reduction of the conductance to K+, like that seen in CHCl3, would make the repolarizing phase of the action potential slower, and the action potential would broaden. A similar result would be expected with halothane, which reduces the conductance proper. Conversely, the membrane would repolarize at a much faster rate with an increased conductance and a reduction of the rate at which channels close, as would occur in the case of the noninactivating channels exposed to isoflurane. Changes in the K+ conductance also affect the onset of the action potential; the firing threshold is reached earlier when the conductance is decreased and is delayed when it is increased. All of the above effectively change the firing frequency and conduction velocity of
the cell. As mentioned above, A-type currents also play important roles in excitability and action potential propagation. Depending on the resting potential of a neuron, A-type currents will influence the duration and repolarization rate of the action potentials or influence the latency between action potentials and, consequently, the firing frequency. In the latter scenario, which occurs in neurons with depolarized resting potentials, a higher conductance and longer times in the open state would lengthen the period between action potentials, effectively reducing the firing frequency. A reduced conductance or shorter openings, on the other hand, would speed this process. The rates at which these changes occur and the voltage dependence of the processes involved are governed by the gating mechanisms of the channels, and, as is clearly illustrated in this study, general anesthetics can have a dramatic influence on the gating process of K⁺ channels.

Although there is a large body of evidence for volatile anesthetic block of K⁺ channels in native cells as well as in expressed recombinant channels, most of the reports concern anesthetic effects on other types of K⁺ channels (9, 17, 29). In general, CHCl₃, halothane, and isoflurane depress currents through most K⁺ channels. However, it is also known that at low concentrations volatile anesthetics can produce hyperexcitability, whereas at higher concentrations, still within the clinically relevant range, there is depression (8, 18, 29). This type of behavior could be explained by differential modulation of the various types of K⁺ channels in cell bodies, dendrites, and axons (8). There is, however, precedent for activation of K⁺ currents induced by volatile anesthetics. In Aplysia neurons, Winogar et al. (31) found that halothane and isoflurane induced an increase in the probability of opening of a resting K⁺ channel, the S channel, which hyperpolarized the resting membrane potential, producing a loss of excitability. The inhibition by CHCl₃ and halothane and the stimulation by isoflurane seen in the present study are, therefore, consistent with previous observations in isolated cells and axons.

In a comparative study with different clones of K⁺ channels, Zorn et al. (32) reported block by halothane of ShakerB (ShB1) currents expressed in Xenopus oocytes at concentrations similar to those used in the present study. The peak current was reduced, and no change in channel inactivation was observed. In another study with recombinant channels, Vener et al. (30) reported block of peak and steady-state conductance and increased rates of activation and inactivation of the Shaker analog RCK2 (Kv1.6) treated with halothane (0.5–8%). The results presented in this paper are in line with the inhibitory effect of halothane on channel conductance seen in the ShB1 (32) and in the RCK2 (30), but, in contrast to the RCK2, halothane had little or no influence on channel kinetics of any of the clones studied, as in the ShB1.

In conclusion, Shaker-like K⁺ channels are differentially modified by the action of general anesthetics. The evidence points toward a direct interaction of the anesthetics with the channel protein as opposed to an indirect effect due to perturbation of the bilayer milieu.

The action of the anesthetics tested involves modification of the channel at transitions near the open state, affecting kinetics, mostly of closing, and channel conductance. On the basis of the data presented here, neural function dependent on the activity of noninactivating Shaker-like delayed rectifying channels would differ substantially depending on the anesthetic. One could predict that halothane and CHCl₃ would produce different firing patterns and repolarization rates than isoflurane. Although there are differences that could be attributed to the nature of the K⁺ channels studied, the data presented in this paper are consistent with that obtained in similar studies in this and other systems at clinically relevant concentrations of volatile anesthetics.

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REFERENCES


