Rapid and Direct Modulation of $\text{GABA}_A$ Receptors by Halothane

Xiaoshen Li, Ph.D.,* Cynthia Czajkowski, Ph.D.,† Robert A. Pearce, M.D., Ph.D.‡

Background: Hypotheses regarding the nature of channel modulation by volatile anesthetics have focused primarily on "membrane actions" of anesthetics and more recently on direct actions of volatile agents on receptor proteins themselves. With the recognition that many channels are subject to modulation by intracellular enzymes, such as protein kinases and phosphatases, and recent demonstrations that the activity of these modulators themselves may be altered by anesthetic agents, a third possibility has been suggested: anesthetic actions on channels may be indirect, produced, for example, via direct effects on intracellular enzyme systems.

Methods: To determine the contribution of indirect versus direct modulation, the authors compared effects of the volatile anesthetic halothane on $\gamma$-aminobutyric acid A receptors under two conditions: in the whole cell configuration with intact intracellular regulatory systems, and in the excised patch configuration, in which intracellular signaling systems have been disrupted. They also evaluated the effects of rapid application and withdrawal of anesthetic to determine the time course of onset and offset of the anesthetic actions on these channels.

Results: Characteristic changes in $\gamma$-aminobutyric acid A receptor function occurred in excised patches as in whole cells, did not require alteration of receptor phosphorylation, and were rapid (onset and offset of anesthetic action occurred within milliseconds).

Conclusions: These results are not consistent with indirect modulation but rather indicate that volatile agents modulate $\gamma$-aminobutyric acid A receptors by direct action on the channel complex or surrounding lipid membrane. (Key words: Anesthetics; electrophysiology; ion channel; patch clamp.)

* Graduate Student, Department of Zoology.
† Assistant Professor, Department of Physiology.
‡ Associate Professor, Departments of Anesthesiology and Anatomy.

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Address reprint requests to Dr. Pearce: Department of Anesthesiology, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706. Address electronic mail to: rapearce@facstaff.wisc.edu

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to phosphorylation, and volatile agents have been shown to alter protein kinase activity in various in vitro assay systems. Similarly, other active second-messenger systems, such as protein phosphatases, G-protein-mediated signaling via direct channel interactions or lipid mediators, calcium signaling, and nitric oxide, may be considered also.

In the current study, we tested the hypothesis that volatile agents alter GABA receptor responses indirectly; i.e., by altering receptor modulation produced by intracellular factors or pathways. This was accomplished by comparing the effects of halothane on receptor deactivation kinetics in the whole cell and excised recording configurations, in both the presence and the absence of adenosine triphosphate (ATP). In addition, we studied how rapidly alterations in receptor function may be brought about by halothane, supposing that indirect actions require some time to develop. Our results indicate that changes in GABA receptor function are direct because anesthetic modulation occurred in excised patches and was observed within milliseconds of anesthetic application.

Materials and Methods

Transient Expression in HEK 293 Cells

HEK 293 cells (American Type Culture Collection CRL 1573, Manassas, VA) were maintained in standard culture conditions (37°C, 5% CO2). The culture medium consisted of minimal essential medium with Earle salts (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Hyclone Laboratories, New Brunswick, NJ). Cells were plated on 12-mm-circle cover glass (Fisher Scientific, Pittsburgh, PA) in 60-mm culture dishes 24 h before transfection. Rat GABA receptor subunits (α1, β2, γ2s, and CD8 cDNA) were subcloned into the multiple cloning site of a mammalian expression vector (pCEP4; InVitrogen, Carlsbad, CA) for transient transfection of HEK 293 cells. Cells were cotransfected at 10–20% confluence with pCEP-α1, pCEP-β2, pCEP-γ2s, and pCEP-CD8 at a 1:1:1:1 ratio (0.3 μg/subunit) using polyamine reagent Trans-LT2 (PanVera, Madison, WI). Recordings were performed 24–48 h after transfection. A bead-labeling technique using the cytokine receptor CD8 was used to identify cells transiently transfected. No systematic differences in GABA receptor kinetics or modulation were observed with the time in culture or with the bead-labeling technique.

Electrophysiologic Recordings

Recordings were performed at room temperature on the stage of an inverted Nikon microscope (Melville, NY) with Hoffman optics. Before recording, antibody-coated beads, Dynal M-450 CD8 (Dynal, Lake Success, NY), were added to the culture dish at 1:1,000 dilution. After a 5-min incubation, the coverslip was transferred to the recording chamber. Cells decorated with beads, indicating a high level of exogenous protein expression, were chosen for study. For whole cell experiments, only the smallest individual cells were used to maximize mechanical stability and minimize solution exchange times using rapid application techniques. After obtaining stable whole cell access, negative holding pressure was applied to aid mechanical stability, and the cell was lifted from the coverslip and positioned in front of the multibarrel solution exchange pipette. For excised outside-out patch recordings, coverslips were coated with poly-D-lysine to facilitate adherence of the cell to the coverslip and thus permit excision.

Recording electrodes were fabricated from KG-33 glass (Garner Glass Company, Claremont, CA) using a multistage puller (Flaming-Brown model P-87; Sutter Instruments, Novato, CA) and coated with Sylgard (Dow-Corning Company, Midland, MI) to reduce electrode capacitance. The tips were not fire-polished routinely. Open-tip electrode resistance was typically 2–4 MΩ when filled with standard recording solution. All recordings were obtained at a holding potential of −40 mV using a low-noise patch amplifier (Axopatch 200A; Axon Instruments, Foster City, CA). Access resistance, typically 4–10 MΩ, was compensated by 70–80% using amplifier circuitry. Seal resistance for patches or access resistance and capacitance for whole cell recordings were monitored during the course of the experiments using amplifier circuitry. Data acquisition and display were performed using pCLAMP6 (Axon Instruments).

Rapid Solution Exchange Technique

Solutions were applied to whole cells and excised outside-out patches using a gravity-fed multibarrel application pipette mounted to a piezoelectric stacked translator (model P-245.50; Physik Instrumente, Costa Mesa, CA). For most experiments a two-barrel “theta” pipette was used (fashioned from Thin Theta; Sutter Instruments, Novato, CA), but some later experiments used a three-barrel linear array of glass tubing (Vitrocom, Mountain Lakes, NJ). The voltage input to a high-voltage amplifier (model P-270; Physik Instrumente) used to drive the stacked translator was filtered using an eight-
pole Bessel filter (model 902LPF; Frequency Devices, Haverhill, MA) to reduce oscillations arising from rapid acceleration of the pipette.

Whole cells or excised outside-out patches were lifted above the glass coverslip and positioned near the interface between flowing solutions, approximately 100 μm from the end of the application pipette. The solution exchange time was estimated initially by observing the recording electrode open-tip liquid junction current during the switch from standard to diluted saline. The exchange speed could be controlled by increasing the height of the solution reservoirs. There was a trade-off, however, in that faster exchanges arising from greater solution velocity led to mechanically less stable recordings. For whole cell recordings, reservoirs typically were adjusted to yield open-tip exchange times of approximately 500 μs (solution velocity approximately 60 μm/ms), which produced an acceptably stable configuration if small cells were chosen.

The open-tip method for estimating solution exchange times has been found by other investigators to correspond well to more direct methods of measuring exchange rates for excised outside-out patches, using changes in ionic currents across intact patches in response to changes in ionic driving forces. Solution exchanges for whole cell recordings, however, should be expected to be slower than these estimates indicate because of fluid dynamic considerations. To obtain a more direct measure of the whole cell solution exchange time, we performed separate experiments to measure the change in endogenous potassium current in the whole cell configuration in response to changing ionic conditions (fig. 1). For these experiments, endogenous potassium currents were elicited by depolarizing from a holding potential of −60 to −20 mV. With 140 mM potassium in the pipette solution, as the extracellular solution was switched from 5 mM to 20 mM potassium, the driving force for potassium was reduced by 20%, leading to a reduction in the activated potassium current. The rate of current change was used as an indication of whole cell solution exchange time. For rapid solution flows that produced open-tip exchanges of approximately 100 μs, whole cell exchanges as rapid as 0.5 ms were observed (fig. 1). For slower flows leading to 500-μs open-tip exchanges, whole cell exchange was approximately 2 ms. Therefore, even for whole cell recordings (which are analogous to the “nucleated patch” recording configuration), exchange times were adequate to test for changes in receptor kinetics and onset of anesthetic effects using a time scale measured in milliseconds.

**Solutions and Drugs**

The recording chamber was perfused continuously with HEPES-buffered saline containing the following: NaCl: 135 mM; KCl: 5.4 mM; MgCl₂: 1.0 mM; CaCl₂: 1.8 mM; and HEPES: 5.0 mM, at a pH of 7.2. This standard saline also was used as a control solution in the rapid application pipette. Recording pipettes were filled with the following: CsCl: 140 mM; Na-HEPES: 10 mM; ethylene glycoltetraacetic acid (EGTA): 10 mM; and Mg-ATP: 1 mM, at a pH of 7.3. ATP was omitted from the pipette.
solution for some experiments. GABA was prepared as a 1-mM stock solution in standard saline and diluted to achieve the desired concentration.

For anesthetic application, solution reservoirs were bubbled continuously with 0.8% halothane using a calibrated vaporizer, which was indicated by preliminary experiments to produce a near maximal effect on deactivation kinetics. The gas-phase concentration was monitored throughout the experiments using a gas monitor (Multigas Monitor 602; Criticare Systems, Waukesha, WI). This gas-phase concentration corresponds to a liquid-phase concentration of 0.43 mM, or 1.6 times the minimum alveolar concentration. The perfusion system was constructed of Teflon (Cole-Parmer, Vernon Hills, IL) and glass to prevent the loss of the anesthetic. All chemicals were obtained from Sigma (St. Louis, MO). Distilled water was used for preparation of all solutions. Halothane was obtained from Halocarbon Laboratories (River Edge, NJ).

Data Analysis

Current deactivation was fit by exponential functions, beginning shortly after the peak of the response, using a Levenberg–Marquardt algorithm (Origin 5.0; Microcal Software, Northampton, MA). During the fitting process, the goodness of fit was evaluated by the chi-square analysis, and adequacy of fit to biexponential function was judged by eye. For some recordings, primarily in the whole cell configuration, the onset of the deactivation phase showed an obvious sigmoidal waveform lasting more than 5 ms. This probably resulted from slow solution exchange; therefore, these recordings were not used for quantitative analysis. From the values obtained by fitting, a weighted time constant of decay $\tau_{\text{decay}}$ was calculated ($\sum \tau_i A_i / \sum A_i$).

To evaluate the concentration–response characteristics of different preparations, peak currents during prolonged application of GABA (100–400 ms) were plotted as a function of agonist concentration. Peak current amplitude was normalized to the response at a saturating agonist concentration (1–10 mM) and fitted to the Hill equation:

$$i = 1 - \frac{1}{1 + \left(\frac{[\text{GABA}]}{\text{EC}_{50}}\right)^n}$$

where EC50 is the concentration that yields a half-maximal response, and n is the Hill coefficient.

Results

Whole Cell versus Excised Patch

Comparison of Baseline Responses. $\gamma$-Aminobutyric acid application (1 mM, 1–4 ms) to whole cells and outside-out excised patches produced inhibitory postsynaptic currents (IPSCs) “simulated” with characteristics similar to simulated in situ, including rapid activation and deactivation that extended well beyond the period of agonist application (fig. 2). No significant differences were seen between whole cell and excised patch responses in decay time constants, proportions of the components, or the weighted decay time constant ($[\tau]_{\text{decay}}$) (fig. 2B). Peak responses to more prolonged applications of agonist (100–400 ms) were also similar in the two configurations (fig. 2C). On average, EC50 did not differ in whole cell (EC50 = 30.6 ± 15.2 μM; Hill coefficient = 1.28 ± 0.38; n = 5) and excised-patch recordings (EC50 = 42.5 ± 15.4 μM; Hill coefficient = 0.99 ± 0.44; n = 3; P > 0.10, Student t test). Patch excision was not found to alter these receptor properties; therefore, comparisons of anesthetic actions in the two configurations should not be confounded by altered baseline receptor properties.

Comparison of Halothane Effects. The effects of halothane on agonist-induced responses were tested to determine whether the ability of halothane to prolong the simulated inhibitory postsynaptic currents was altered or lost after patch excision. For these experiments, baseline responses were obtained, and solutions in control and GABA barrels were switched to solutions that contained halothane. Complete exchange took approximately 6–10 s, and the first responses in the presence of halothane were obtained after an additional wait of approximately 15 s. After obtaining two to four responses (which were averaged for analysis), solutions were switched back and “wash” responses were obtained. In whole cell and excised patch recordings, halothane

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(0.43 mM) slowed current deactivation more than two-fold and reduced the peak amplitude of the response by approximately 20% (fig. 3; table 1). Slowed decay resulted from an increase in the time constant and proportion of the slow component but no change in the time constant of the fast component (table 1).

Fig. 3. Effect of halothane on deactivation kinetics: comparison of whole cell and excised receptor responses. (A) Response to a brief pulse of γ-aminobutyric acid, with 1 mM adenosine triphosphate in the recording pipette. (Top) Shows the liquid junction current recorded at the end of the experiment. Halothane 0.43 mM reduced the peak current and prolonged the deactivation phase. Both effects were reversible during wash. (B) Normalized responses (Ipeak/Ipeak,control), for comparison of deactivation kinetics. (C) Summary of effects on deactivation kinetics (τdecay). **P < 0.01, n = 6 for whole cells and excised patches.

Halothane Effects in the Absence of Adenosine Triphosphate

Because membrane- or channel-associated kinases or phosphatases may be maintained after excision, we considered whether halothane may have altered channel activity indirectly via a change in phosphorylation mediated by tightly associated enzymes. This possibility was evaluated by investigating the response in the absence of the phosphorylation substrate ATP (fig. 4). Again, for
Table 1. Effect of Halothane on Receptor Kinetic Properties

<table>
<thead>
<tr>
<th>Recording Condition</th>
<th>Control</th>
<th>Halothane</th>
<th>Wash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM ATP n = 6</td>
<td>$\tau_1 = 10.7 \pm 3.1$</td>
<td>$\tau_1 = 12.7 \pm 5.4$</td>
<td>$\tau_1 = 11.9 \pm 4.0$</td>
</tr>
<tr>
<td>% Slow</td>
<td>29.7%</td>
<td>61.9%</td>
<td>29.3%</td>
</tr>
<tr>
<td>$\tau_{\text{decay}}$</td>
<td>23.1%</td>
<td>20.9%</td>
<td>21.1%</td>
</tr>
<tr>
<td>Peak</td>
<td>83.5%</td>
<td>83.4%</td>
<td>91.4%</td>
</tr>
<tr>
<td>Excised patch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM ATP n = 6</td>
<td>$\tau_1 = 10.5 \pm 4.9$</td>
<td>$\tau_1 = 13.9 \pm 10.2$</td>
<td>$\tau_1 = 9.5 \pm 3.3$</td>
</tr>
<tr>
<td>% Slow</td>
<td>45.5%</td>
<td>60.3%</td>
<td>53.5%</td>
</tr>
<tr>
<td>$\tau_{\text{decay}}$</td>
<td>27.2%</td>
<td>16.7%</td>
<td>24.5%</td>
</tr>
<tr>
<td>Peak</td>
<td>77.1%</td>
<td>70.5%</td>
<td>88.7%</td>
</tr>
<tr>
<td>Whole cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ATP n = 5</td>
<td>$\tau_1 = 14.7 \pm 5.2$</td>
<td>$\tau_1 = 14.9 \pm 4.0$</td>
<td>$\tau_1 = 13.3 \pm 5.6$</td>
</tr>
<tr>
<td>% Slow</td>
<td>31.3%</td>
<td>67.5%</td>
<td>60.0%</td>
</tr>
<tr>
<td>$\tau_{\text{decay}}$</td>
<td>31.3%</td>
<td>34.8%</td>
<td>31.1%</td>
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<tr>
<td>Peak</td>
<td>76.1%</td>
<td>75.8%</td>
<td>86.4%</td>
</tr>
<tr>
<td>Excised patch</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 ATP n = 5</td>
<td>$\tau_1 = 12.8 \pm 3.0$</td>
<td>$\tau_1 = 24.9 \pm 10.6$</td>
<td>$\tau_1 = 17.7 \pm 5.4$</td>
</tr>
<tr>
<td>% Slow</td>
<td>52.3%</td>
<td>58.5%</td>
<td>48.5%</td>
</tr>
<tr>
<td>$\tau_{\text{decay}}$</td>
<td>49.1%</td>
<td>34.8%</td>
<td>41.5%</td>
</tr>
<tr>
<td>Peak</td>
<td>61.5%</td>
<td>59.6%</td>
<td>60.5%</td>
</tr>
</tbody>
</table>

All values mean ± SD.

* $P < 0.05$.
† $P < 0.01$ versus control. Student $t$ test.
‡ $P < 0.01$ versus excised patch (control, + ATP). Student–Newman–Keuls test.

$\tau_1$, $\tau_2$ = fast and slow deactivation time constants for biexponential fits (ms); $\tau_{\text{decay}}$ = weighted time constant (ms); peak = $100 \times (p_{\text{peak}}/p_{\text{peak(control)}})$.

whole cell recordings, halothane increased the time constant and proportion of the slow component but did not change the fast component (table 1). In excised patches, a slightly different pattern of change was seen. Baseline responses were slower compared with excised patches with ATP included in the recording pipette, and only the slow time constant was increased, without a change in the proportion of the two components. Nevertheless, the degree of prolongation of the decay time was not different from that for excised patches with ATP ($P > 0.05$, Student $t$ test). From these results, we conclude that prolongation of current decay and reduction in peak amplitude by halothane are not produced through alteration of receptor phosphorylation.

**Rate of Channel Modulation by Halothane**

Although the ATP-free results described effectively rule out alteration in phosphorylation state, other possible indirect actions might remain even in excised patches; for example, direct G-protein receptor coupling or lipid mediator-based modulation. It is likely that such pathways would take substantial time to effect a change in GABA receptor function. For example, G-protein-coupled receptor activation proceeds with a delay of some tens of milliseconds, even for direct coupling. Indirect coupling, produced by generation of a lipid product or other second messenger, would be expected to take even longer.

To assess the possibility that some other indirect pathway may be involved in channel modulation, we tested the speed with which alterations in channel function can be brought about by halothane. Two types of experiments were performed. In the first set, we measured the speed with which channel activation at low agonist concentrations is altered by anesthetic. In the second set, we measured the rate of change in channel deactivation and current block after anesthetic application and withdrawal.

**Responses at Low Agonist Concentrations**

Brief pulses of low concentrations of agonist led to slow channel activation that continued until the end of the pulse and deactivation that had biexponential decay kinetics (fig. 5). Halothane caused the deactivation to be...
slowed, as was also observed for high-concentration responses. In this case, however, the peak amplitude was increased to 155.0 ± 63.7% of the control value (n = 7).

We used this increase in current amplitude during low-concentration activation to test how quickly halothane can alter channel activation. For these experiments, pulses of 10 μM GABA, 10 μM GABA plus halothane, or 10 μM GABA in the continuous presence of halothane were applied during whole cell recordings, and the rate of channel activation was compared. An example of one such experiment is shown in figure 6. The peak current was found to be larger with coapplication of GABA and halothane, and with application of GABA in the continuous presence of halothane (fig. 6).

Fig. 6. Rapid onset of current enhancement by halothane. Whole cell responses to 10 μM γ-aminobutyric acid (GABA) were obtained under control conditions (without halothane, trace i), with application of halothane, and with GABA in the continuous presence of halothane (trace ii and iii). Inset shows the current rising phase on an expanded time scale.

Evaluating using an expanded time scale, the rising phase is seen to be altered within 2 ms of halothane application (fig. 6) and is indistinguishable for 10 μM GABA co-applied with halothane and 10 μM GABA in the continuous presence of halothane. Therefore, the onset of halothane action is extremely rapid. At the discontinuation of

Fig. 5. Effect of halothane on responses with low concentrations of γ-aminobutyric acid (GABA; 10 μM). (A) Halothane slowed deactivation in response to a brief pulse (5 ms). Unlike with a saturating concentration of agonist, the peak response was increased. (B) Response to prolonged application of GABA (300 ms). Halothane accelerated activation and slowed deactivation.

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GABA and halothane coapplication, a prominent off-response, or "surge current," was observed, as seen by other investigators after sevoflurane and barbiturate application. It has been postulated that this current represents the removal of superimposed open channel block by the anesthetic.

**Responses at High Agonist Concentrations**

The experiments described to this point showed that the onset of halothane enhancement of low-concentration responses is rapid and therefore unlikely to be produced by an indirect mechanism. To assess directly the speed with which changes in deactivation kinetics per se can be altered and how quickly such effects can be reversed, we performed experiments using a rapid-application device with three barrels. This allowed us to expose the cell to halothane only after the withdrawal of agonist to observe the rate at which the anesthetic slows deactivation. We found that if halothane was applied to the cell 200 ms after discontinuation of GABA application, current was reduced and deactivation was slowed in the presence of the anesthetic (figs. 7A and B). The rapid onset and removal of block (fig. 7C) precluded accurate determination of the rates of change in deactivation, but clearly the changes in deactivation and block occur within milliseconds of anesthetic application, providing further evidence that halothane acts directly to alter the kinetics of channel deactivation.

**Discussion**

The primary findings of this study are that the effects of halothane on GABA receptors are similar in excised receptors and in whole cells, and that these effects occur within milliseconds of anesthetic application. We conclude that the actions of halothane do not result from indirect modulation via cellular signaling systems, but rather from a direct action on the receptor complex or surrounding membrane.

To reach this conclusion, we relied on the exclusion of possible indirect pathways using various approaches. Removal of receptors from the influence of soluble intracellular signaling systems via patch excision depends on the dilution of soluble factors in the pipette solution after excision. Although we have no direct evidence that such dilution is adequate to reduce the concentrations of all factors to levels low enough to prevent any possible influence, this assumption seems quite reasonable. In any case, the more concerning possibility that membrane-bound components may survive the process of excision necessitated that we consider indirect modulation even in excised patches.

For phosphorylation, we were able to exclude indirect channel modulation by removing the substrate ATP from the pipette solution, in which case halothane continued to prolong current decay in whole cell and in excised-patch recordings (fig. 4). Because the effect of halothane not only remained, but was reversible in the absence of ATP, we are able to exclude indirect actions via both protein kinases and phosphatases. For calcium, which has been proposed as a direct GABA receptor modulator and a mediator of halothane action on the GABA receptor, to produce altered levels even in excised patches would necessitate a calcium source that survives excision and is not overcome by 10 mM EGTA in the pipette solution. Because we did not detect transmembrane calcium currents, and possible membrane-associ-
at ed vesicular stores should be depleted quickly in the excised configuration, indirect alteration \textit{via} calcium ions also was ruled out effectively.

To exclude other possible indirect pathways, such as membrane-delimited signaling \textit{via} direct G-protein-channel interactions, we observed the speed with which alterations in receptor properties can be altered by halothane. We observed that full effects were achieved within approximately 2–10 ms of anesthetic application. The onset of anesthetic action may be substantially faster even than this, because several milliseconds were necessary to detect current at intermediate concentrations of GABA because of its slow activation (fig. 6), and, at high GABA concentration, the rapid onset of superimposed block obscured visualization of deactivation kinetic changes (fig. 7). Using the time course of GABA\textsubscript{A} inward rectifier potassium current as a guideline to estimate the time necessary for direct G-protein–channel interaction, it has been found that current onset occurs with a lag of 10—20 ms, with approximately 12–16 ms thought to be necessary for G-protein activation.\textsuperscript{22} Thus, the rapid action of anesthetic on deactivation kinetics argues against this membrane-delimited indirect pathway and others that were not manipulated directly in these experiments.

The recent finding that mutation of individual amino acid residues within the TM2 and TM3 transmembrane segments can produce receptors in which activity is not enhanced by the volatile agents enflurane or isoflurane\textsuperscript{26} is consistent with a direct action of volatile agents on the GABA\textsubscript{A} receptor. Although it has not been determined whether the mutated residues are part of an anesthetic binding pocket or are elements of a transduction pathway, the results of the current experiment indicate that if the latter is the case, the mutations are not simply disrupting an indirect modulatory pathway.

\textit{Role of Indirect Modulation in Anesthetic Action}

These results indicate that, at least for the GABA\textsubscript{A} receptor, indirect alteration \textit{via} a modulatory pathway is not necessary for halothane to alter channel kinetics. This is not to say, however, that indirect modulation does not occur, the net change reflecting actions \textit{via} indirect and direct pathways. For example, activation of protein kinase or inhibition of protein phosphatase may increase channel phosphorylation and speed channel deactivation,\textsuperscript{12} partially counteracting the direct prolonging action. We observed that baseline kinetics in the absence of ATP were slower for excised patches and that, for whole cell recordings, the degree of prolongation was greater in the absence of ATP (fig. 3 vs. fig. 4), though this difference did not reach statistical significance. Because the degree of prolongation was comparable with and without ATP and in whole cells and excised receptors, however, such an indirect effect does not appear to be great for these expressed receptors. Nevertheless, we tested effects on only a single combination of subunits, and the cells used for these studies were derived from nonneuronal tissue. It is possible that indirect actions \textit{via} second-messenger modulation may be more important for other subunit combinations or in neurons with a different complement of regulatory proteins.

\textit{Implications for the “Anesthetic Binding Site”}

A primary motivation for the current study was to try to explain why various structurally unrelated receptors and channels are affected by volatile anesthetics. This led us to consider the possibility that a factor that sensitive channels have in common is modulation by some intracellular signaling system that regulates diverse proteins, such as phosphorylation or calcium. Some component of such a system then would be the primary anesthetic target, a specificity of drug–target interaction would be maintained, and indirect modulation would help to explain the diversity of actions.

Because our results indicate that this does not seem to be the case, what are the implications regarding anesthetic–target interactions? A classic “lock-and-key” model of drug–protein interactions does not seem likely, given the variety of structurally dissimilar anesthetics and channels that interact. Nevertheless, some hydrophobic or amphiphilic region or regions of sensitive channels may be the sites to which anesthetics bind to exert their actions. This “protein-binding” model is certainly compatible with our current results, as it is with studies of aqueous-soluble protein effects\textsuperscript{3} and with GABA\textsubscript{A} receptor mutation studies.\textsuperscript{26} Although our results do not exclude the possibility that the anesthetic binding site is a closely associated regulatory protein that binds to the GABA\textsubscript{A} receptor subunits that make up the channel, such a protein would be considered to be a component of the receptor complex by conventional definition, so our conclusion that the anesthetic action is direct must include this possibility.

It is also possible that anesthetics exert their effects \textit{via} a lipid-membrane site, or at the interface between lipid and protein.\textsuperscript{10} This not only is suggested by the well-known correlation between anesthetic potency and lipid solubility, but also is consistent with the lack of...
saturation of enhancement recently reported for the nicotinic acetylcholine receptor. The current results do not help to distinguish among direct protein, lipid, and interface sites, each of which would appear as “direct” in our experiments and could have as quick an onset as was observed. Nevertheless, these results provide reassurance that future studies to identify the appropriate class of biophysical sites (protein, lipid, or interface), and eventually to identify functionally active anesthetic binding sites, need not focus on interactions with intracellular modulatory components, but may proceed in reduced systems such as the excised patch or possibly reconstituted channel proteins.

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References

4. Zimmerman SA, Jones MV, Harrison NL: Potentiation of GABA<sub>A</sub> receptor Cl<sup>-</sup> current correlates with in vivo anesthetic potency. J Pharmacol Exp Ther 1994; 270:987–91
5. Gage PW, Robertson B: Prolongation of inhibitory post-synaptic currents by pentobarbitone, halothane and ketamine in CA1 pyramidal cells in rat hippocampus. Br J Pharmacol 1985; 85:675–81
27. Raines DE, Zachariah VT: Isoflurane increases the apparent agonist affinity of the nicotinic acetylcholine receptor. Anesthesiology 1999; 90:135–46

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