

# Nonhalogenated Alkane Anesthetics Fail to Potentiate Agonist Actions on Two Ligand-gated Ion Channels

Douglas E. Raines, M.D.,\* Robert J. Claycomb, B.S.,† Michaela Scheller, M.D.,‡ Stuart A. Forman, M.D. Ph.D.\*

**Background:** Although ether, alcohol, and halogenated alkane anesthetics potentiate agonist actions or increase the apparent agonist affinity of ligand-gated ion channels at clinically relevant concentrations, the effects of nonhalogenated alkane anesthetics on ligand-gated ion channels have not been studied. The current study assessed the abilities of two representative nonhalogenated alkane anesthetics (cyclopropane and butane) to potentiate agonist actions or increase the apparent agonist affinity of two representative ligand-gated ion channels: the nicotinic acetylcholine receptor and  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor.

**Methods:** Nicotinic acetylcholine receptors were obtained from the electroplax organ of *Torpedo nobiliana*, and human GABA<sub>A</sub> receptors ( $\alpha_1\beta_2\gamma_2$ ) were expressed in human embryonic kidney 293 cells. The *Torpedo* nicotinic acetylcholine receptors apparent agonist affinity in the presence and absence of anesthetic was assessed by measuring the apparent rates of desensitization induced by a range of acetylcholine concentrations. The GABA<sub>A</sub> receptor's apparent agonist affinity in the presence and absence of anesthetic was assessed by measuring the peak currents induced by a range of GABA concentrations.

**Results:** Neither cyclopropane nor butane potentiated agonist actions or increased the apparent agonist affinity (reduced the apparent agonist dissociation constant) of the *Torpedo* nicotinic acetylcholine receptor or GABA<sub>A</sub> receptor. At clinically relevant concentrations, cyclopropane and butane reduced the apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization induced by low concentrations of agonist.

**Conclusions:** Our results suggest that the *in vivo* central nervous system depressant effects of nonhalogenated alkane anesthetics do not result from their abilities to potentiate agonist actions on ligand-gated ion channels. Other targets or mechanisms more likely account for the anesthetic activities of nonhalogenated alkane anesthetics.

THE *Torpedo* nicotinic acetylcholine receptor (nAChR),  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>R), the glycine receptor, and 5-hydroxytryptamine<sub>3</sub> receptor together form an anesthetic-sensitive superfamily of ligand-gated ion channels (LGICs) that are critical for synaptic function.<sup>1,2</sup> Members of this superfamily are widely considered to be the most important targets of general anesthetics and are frequently used in studies of anesthetic molecular mechanisms.<sup>3-11</sup> Such studies have

shown that, at clinically relevant concentrations ( $\leq 3$  minimum alveolar concentration [MAC]), general anesthetics representing a wide range of chemical classes (including halogenated alkanes, halogenated and nonhalogenated ethers, and alcohols) increase ion flux or apparent desensitization rates induced by low agonist concentrations. When experiments have been performed using a range of agonist concentrations, this action has been shown to reflect a reduction in the receptor's apparent agonist dissociation constant ( $K_d$ ) (an increase in apparent agonist affinity).<sup>3,8,12-15</sup>

Despite a large body of literature documenting the effects of anesthetics on a variety of LGICs, there have been no studies to assess the effects of nonhalogenated alkane anesthetics on any LGIC. Nonhalogenated alkanes, in particular cyclopropane, were commonly used in the past as clinical inhalational anesthetics before they were supplanted by nonflammable halogenated analogs. The goal of the current study was to test the hypothesis that agonist potentiation of LGICs might also play an important role in the clinical actions of nonhalogenated alkane anesthetics. This was achieved by assessing the abilities of cyclopropane and butane to increase the apparent agonist affinities of the two best characterized LGICs: the nAChR and the GABA<sub>A</sub>R. Our results reveal that, in contrast to nearly all other previously studied anesthetics, cyclopropane and butane do not potentiate agonist actions on LGICs at clinically relevant concentrations. This suggests that other molecular mechanisms account for the *in vivo* anesthetic activities of nonhalogenated alkane anesthetics.

## Materials and Methods

*Torpedo nobiliana* was obtained from Biofish Associates (Georgetown, MA). Diisopropylfluorophosphate and acetylcholine were purchased from Sigma Chemical Co. (St. Louis, MO). Alkane anesthetics were purchased from Aldrich (Milwaukee, WI). The fluorescent partial agonist, [1-(5-dimethylaminonaphthalene)sulfonamido] n-hexanoic acid  $\beta$ -(*N*-trimethylammonium bromide) ethyl ester (Dns-C<sub>6</sub>-Cho), was synthesized according to the procedure of Waksman *et al.*<sup>16</sup>

### Preparation and Characterization of Nicotinic Acetylcholine Receptor-rich Membranes

Receptor membranes were obtained from freshly dissected *T. nobiliana* electric organs and prepared using sucrose density gradient centrifugation as described by Braswell *et al.*<sup>17</sup> and in accordance with guidelines es-

\* Assistant Professor of Anaesthesia, Harvard Medical School, Assistant Anesthetist, Massachusetts General Hospital. † Research Assistant, Massachusetts General Hospital. ‡ Anesthesia Resident, Klinik fuer Anaesthesiologie, Klinikum rechts der Isar, Technische Universitaet Muenchen, Munich, Germany, and Research Fellow, Massachusetts General Hospital.

Received from the Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, Massachusetts. Submitted for publication November 16, 2000. Accepted for publication February 16, 2001. Supported in part by grants No. R29-GM53481 and K21-AA00206 from the National Institutes of General Medical Sciences, Bethesda, Maryland, and the Max Kade Foundation, New York, New York.

Address reprint requests to Dr. Raines: Department of Anesthesia, Massachusetts General Hospital, 32 Fruit Street, Boston, Massachusetts 02114. Address electronic mail to: DRaines@partners.org. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

established by the Massachusetts General Hospital Animal Care Committee (Boston, MA). Membranes were stored at  $-80^{\circ}\text{C}$  in *Torpedo* physiologic solution (250 mM NaCl, 5 mM KCl, 3 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 0.02% NaN<sub>3</sub>, pH 7.0) and thawed on the day of use. Acetylcholinesterase activity was inhibited by exposing membranes to 1.0 mM diisopropylfluorophosphate for 60 min before dilution with *Torpedo* physiologic solution to the desired receptor concentration. The number of agonist binding sites was determined from Dns-C<sub>6</sub>-Cho titrations as previously described.<sup>18</sup>

#### *Determination of the Apparent Rate of Acetylcholine-induced Desensitization*

The apparent rate of agonist-induced desensitization was determined with a double-agonist pulse assay using a sequential mixing stopped-flow spectrofluorometer (Applied Photophysics, Leatherhead, United Kingdom).<sup>8</sup> In brief, receptor-rich membranes were first preincubated with acetylcholine for periods ranging from 15 ms to several minutes. The number of receptors able to be activated (nondesensitized) that remained after this preincubation period was then quantified from the amplitude of the rapid fluorescence signal observed when the membrane-acetylcholine solution was rapidly mixed with an assay solution containing the fluorescent partial agonist Dns-C<sub>6</sub>-Cho and a high, channel-activating concentration of acetylcholine (5 mM). In each experiment, receptor membranes (0.8- $\mu\text{M}$  agonist binding sites) were loaded into one of the spectrofluorometer's gas-tight premix syringes, and acetylcholine was loaded into the other gas-tight premix syringe. The solutions were rapidly mixed (1 ms mixing time; 1:1 vol:vol) and allowed to preincubate for the desired time. The nAChR-acetylcholine solution was then mixed (1 ms mixing time; 1:1 vol:vol) with an assay solution containing 10 mM acetylcholine and 20  $\mu\text{M}$  Dns-C<sub>6</sub>-Cho, and the fluorescence emission was recorded. Where appropriate, solutions also contained anesthetic at the desired concentration. Solutions remained within a closed system made of inert materials (glass and Teflon), and anesthetic loss during experiments was negligible as determined by gas chromatographic analysis. An excitation wavelength of 290 nm was provided by a 150-W xenon arc lamp, and the monochromator bandpass was 5 nm. Fluorescence emission greater than 500 nm was measured through a high-pass filter. Fluorescence intensity was recorded for 500 ms after the second mixing step. All experiments were performed at  $20 \pm 0.3^{\circ}\text{C}$ .

#### *Cell Culture and Transfection with Human $\gamma$ -Aminobutyric Acid Type A Receptor cDNAs*

Human embryonic kidney cells (HEK 293; American Type Culture Collection, Rockville, MD) were cultured in minimum essential medium Eagle (ATCC), supplemented with 10% horse serum, 1% penicillin-streptomycin

(all from Gibco BRL, Life Technologies, Grand Island, NY), and maintained at  $37^{\circ}\text{C}$  in a 5% CO<sub>2</sub> incubator. For transient transfection, the calcium phosphate precipitation technique was used as described by Chen and Okayama.<sup>19</sup> Cells were plated on protamine-coated glass coverslips and transfected with cDNAs for the  $\alpha_1$ ,  $\beta_2$ , and  $\gamma_{2L}$  subunits of human GABA<sub>A</sub>R in pCDM8 vectors with a wt/wt ratio of  $1\alpha:2\beta:5\gamma$ . Cells were cotransfected with an expression plasmid ( $\pi\text{H3-CD8}$ ) for the lymphocyte surface antigen CD8- $\alpha$ , which was supplied by Dr. Gary Yellen (Harvard Medical School, Boston, MA). After transfection, cells were incubated for 36–72 h. Shortly before experiments, cells were incubated briefly with polystyrene microspheres, precoated with anti-CD8 antibody (Dynabeads M-450 CD8, Dynal, Great Neck, NY). Thus, cells expressing CD8 were decorated with beads and could be distinguished from untransfected ones.<sup>20</sup>

#### *$\gamma$ -Aminobutyric Acid Type A Receptor Electrophysiology*

For electrophysiology, the medium was replaced by an extracellular solution with 162 mM NaCl, 5.3 mM KCl, 0.67 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.22 mM KH<sub>2</sub>PO<sub>4</sub>, 15 mM HEPES, 5.6 mM glucose, and 2 mM CaCl<sub>2</sub>, adjusted to a pH of 7.30 with NaOH. Patch pipettes were fire-polished, resulting in open tip resistances of 2–5 M $\Omega$ . Pipettes were filled with intracellular solution containing 140 mM KCl, 2 mM MgCl<sub>2</sub>, 11 mM EGTA, 10 mM HEPES, and 10 mM glucose, adjusted to a pH of 7.30 with KOH. GABA solutions were prepared shortly before the experiments. Recordings were performed at room temperature ( $20$ – $22^{\circ}\text{C}$ ) using standard outside-out or whole cell patch clamp techniques. Patches or cells were clamped to  $-50$  mV during recordings; series resistance was compensated. Currents through the patch clamp amplifier (Axopatch 200A; Axon Instruments, Foster City, CA) were filtered (eight-pole Bessel, 2 kHz) and digitized at 5–10 kHz using commercial software (pClamp 8.0, Axon Instruments). Rapid superfusate switching (0.2–1.2 ms, measured with open pipette junction currents) was performed using a piezo-driven quad tube as previously described.<sup>21</sup> Standard protocols repetitively applied different concentrations of GABA alone or in combination with cyclopropane, butane, isoflurane, or octanol at the desired concentrations as pulses lasting 1.5–2.5 s. When anesthetics were applied, they were applied simultaneously with GABA and without preincubation. A 20-s interval between pulses was used for recovery of channel activity from desensitization. Control currents elicited with GABA alone were checked before and after application of anesthetic. Data were analyzed only if precontrol and postcontrol peak currents differed less than 10%. Cyclopropane and butane were stored in airtight syringes during the experiments. All parts of the application system consist of glass or Teflon tubing material.

### Neuronal Nicotinic and N-methyl-D-aspartate Receptor Studies

*Xenopus* frogs were maintained and treated in accordance with regulations specified by the Massachusetts General Hospital Animal Care Committee (Boston, MA). Methods for oocyte expression of ion channels have been previously described.<sup>22</sup> Capped mRNAs were synthesized *in vitro* from linearized cDNA templates using SP6 or T7 RNA polymerase kits (Ambion, Austin, TX) and purified with affinity beads (BIO-101, Vista, CA). Oocytes were injected with mixtures of mRNAs (20–100 ng) encoding neuronal nAChR subunits  $\alpha_4$  and  $\beta_2$  (wt/wt ratio = 1) or N-methyl-D-aspartate (NMDA) receptor subunits NR1b and NR2a (wt/wt ratio = 1) and incubated at 18°C for 48–96 h. For two-microelectrode electrophysiology, oocytes were placed in a 0.1-ml perfusion chamber at room temperature (20–22°C) and voltage clamped at –50 mV *via* glass electrodes filled with 3 M KCl (0.2–1 M $\Omega$ ). Oocytes expressing neuronal nAChRs were superfused with calcium-free ND-96 (96 mM NaCl, 2 mM KCl, 1 mM BaCl<sub>2</sub>, 0.8 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.6) at 2–3 ml/min, and currents were activated with buffer containing 200  $\mu$ M acetylcholine. Oocytes expressing NMDA receptors were superfused in calcium- and magnesium-free ND-96 (MgCl<sub>2</sub> replaced with BaCl<sub>2</sub>) containing 10  $\mu$ M glycine, and currents were activated with 100  $\mu$ M NMDA. Near the middle of a 60-s agonist pulse, anesthetic-agonist solutions (delivered from closed glass syringe reservoirs *via* Teflon tubing and valves) were introduced for 12–16 s. Currents were digitized at 50–200 Hz and recorded on a personal computer.

### Preparation of Anesthetic Solutions

Saturated solutions of cyclopropane or butane were made by bubbling the gases through the desired buffer solution (25–50 ml) within a 250-ml glass bottle sealed with a Teflon septum. A gas inlet and outlet line passed into the bottle through the septum. During the 1–2-h equilibration period at room temperature, solutions were continuously stirred to achieve a saturated aqueous solution. After equilibration, the outlet line was cleared by aspiration, and the saturated solutions were drawn into a gas-tight syringe that was then connected directly to the spectrofluorometer (for nAChR studies) or application system (for GABA<sub>A</sub>R, neuronal nAChR, and NMDA receptor studies) *via* a Teflon stopcock. In parallel experiments using the same technique, the concentrations of cyclopropane and butane in saturated solutions (10.6 and 1.1 mM, respectively) were determined by gas chromatography to be within 10% of that predicted by their aqueous:gas partition coefficients.<sup>23</sup> Saturated solutions of isoflurane (15 mM) and octanol (4.5 mM) were prepared by mixing excess anesthetic with buffer overnight. To achieve the desired anesthetic concentration,

saturated solutions were diluted with buffer within a gas-tight syringe.

### Conversion of Minimum Alveolar Concentration to Aqueous EC<sub>50</sub>

The approach for converting MAC values into aqueous EC<sub>50</sub> values (and *vice versa*) has been described by Franks and Lieb.<sup>24</sup> All MAC values for inhalational anesthetics are for humans.<sup>23</sup> For butane, MAC was approximated as the EC<sub>50</sub> value reported by Firestone *et al.*<sup>23</sup> For ethanol and octanol, MAC values are for rats.<sup>25</sup>

### Statistical Analysis

Each data point represents the mean of at least three measurements, and the error bars indicate the SD. Statistical analysis was performed using an unpaired Student *t* test and analysis of variance (Prism version 3.0, Graphpad Software, San Diego, CA). *P* < 0.05 was considered significant. Data points on acetylcholine concentration-apparent desensitization rate curves were fit to a Hill equation in the form:

$$k_{\text{app}} = k_{\text{max}} \cdot \left( \frac{[\text{Acetylcholine}]^n}{[\text{Acetylcholine}]^n + (K_d^{\text{app}})^n} \right) \quad (1)$$

where  $k_{\text{app}}$  is the experimentally determined apparent rate of desensitization at each acetylcholine concentration,  $k_{\text{max}}$  is the maximum apparent rate of desensitization induced by high acetylcholine concentrations,  $K_d^{\text{app}}$  is the nAChR's apparent  $K_d$  for acetylcholine, and *n* is the Hill coefficient. The reported errors for all fitted parameters are the SDs derived from the curve fit.

For electrophysiological experiments using the GABA<sub>A</sub>R, an analogous Hill equation was used:

$$I = I_{\text{max}} \cdot \left( \frac{[\text{GABA}]^n}{[\text{GABA}]^n + (K_d^{\text{app}})^n} \right) \quad (2)$$

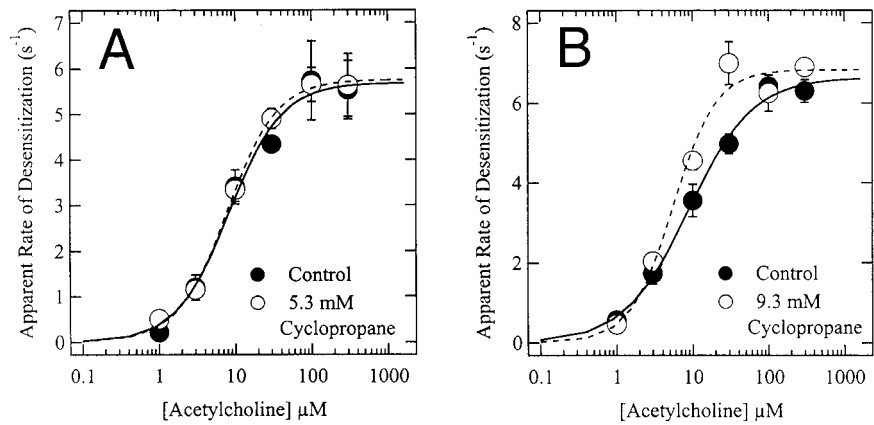
where *I* is the experimentally determined peak current at each GABA concentration,  $I_{\text{max}}$  is the maximal peak current induced by high GABA concentrations,  $K_d^{\text{app}}$  is the GABA<sub>A</sub>R's apparent  $K_d$  for GABA, and *n* is the Hill coefficient. Again, the reported errors for all fitted parameters are the SDs derived from the curve fit.

## Results

### Anesthetic Action on the Nicotinic Acetylcholine Receptor's Apparent Agonist Affinity

In the presence or absence of anesthetic, the apparent rate of *Torpedo* nAChR desensitization increased with acetylcholine concentration before reaching a maximum at high concentrations (fig. 1A). From the acetylcholine concentration dependence of the apparent rate of acetylcholine-induced desensitization, we calculated the receptor's apparent  $K_d$  for this agonist (equation 1). In the absence of anesthetic, *Torpedo* nAChRs in this mem-

**Fig. 1.** The apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization as a function of acetylcholine concentration in the presence and absence of cyclopropane. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 1. (A) The (lack of) effect of 5.3 mM cyclopropane on the apparent rates of acetylcholine-induced desensitization. In the absence of anesthetic (control), the apparent  $K_d$  for acetylcholine in this preparation was  $8 \pm 2 \mu\text{M}$ , the maximum rate of desensitization was  $5.7 \pm 0.3 \text{ s}^{-1}$ , and the Hill coefficient was  $1.2 \pm 0.2$ . The presence of 5.3 mM cyclopropane did not significantly change these values, as the apparent  $K_d$  was  $7.9 \pm 0.5 \mu\text{M}$ , the maximum rate of desensitization was  $5.8 \pm 0.1 \text{ s}^{-1}$ , and the Hill coefficient was  $1.3 \pm 0.1$ . (B) The effect of 9.3 mM cyclopropane on the apparent rates of acetylcholine-induced desensitization. In the absence of anesthetic, the apparent  $K_d$  in this preparation was  $9 \pm 1 \mu\text{M}$ , the maximum rate of desensitization was  $6.6 \pm 0.3 \text{ s}^{-1}$ , and the Hill coefficient was  $1.0 \pm 0.1$ . In the presence of 9.3 mM cyclopropane, the apparent  $K_d$  was  $6 \pm 1 \mu\text{M}$ , the maximum rate of desensitization was  $6.8 \pm 0.4 \text{ s}^{-1}$ , and the Hill coefficient was  $1.5 \pm 0.4$ .

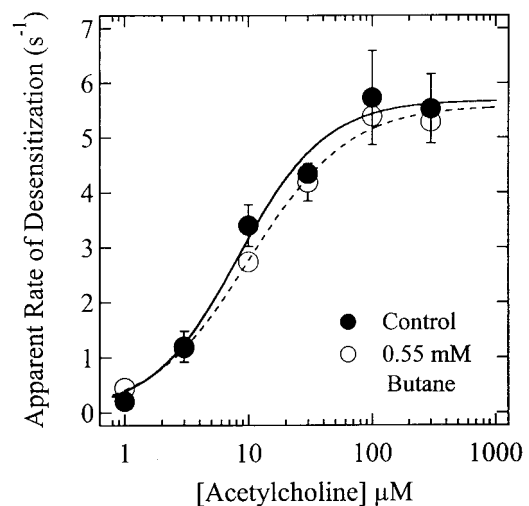


brane preparation had an apparent  $K_d$  for acetylcholine of  $8 \pm 2 \mu\text{M}$  and a maximum rate of desensitization of  $5.7 \pm 0.3 \text{ s}^{-1}$ . Preequilibration with 5.3 mM cyclopropane (a one-half saturated aqueous solution and equivalent to 6.5 MAC) had no effect on either the apparent  $K_d$  for acetylcholine or the maximal rate of acetylcholine-induced desensitization ( $7.9 \pm 0.5 \mu\text{M}$  and  $5.8 \pm 0.1 \text{ s}^{-1}$ ). Figure 1B shows the acetylcholine concentration dependence of the apparent rate of *Torpedo* nAChR desensitization in the absence of anesthetic and in the presence of 9.3 mM cyclopropane (equivalent to 11.4 MAC) using a different receptor preparation. In the absence of anesthetic, *Torpedo* nAChRs in this membrane preparation had an apparent  $K_d$  for acetylcholine of  $9 \pm 1 \mu\text{M}$  and a maximum rate of desensitization of  $6.6 \pm 0.3 \text{ s}^{-1}$ . Preequilibration with 9.3 mM cyclopropane reduced the apparent  $K_d$  for acetylcholine by just 30% to  $6 \pm 1 \mu\text{M}$  (not statistically significant) and had no detectable effect on the maximal rate of desensitization.

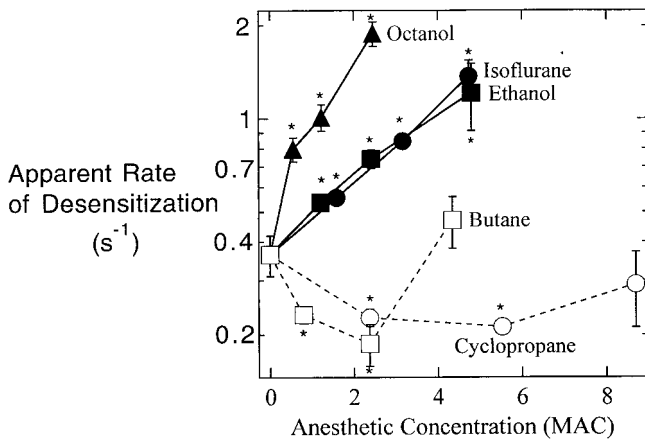
Figure 2 shows the effect of butane on the acetylcholine concentration-response curve for *Torpedo* nAChR desensitization. At a concentration of 0.55 mM (equivalent to 3 MAC), butane failed to reduce the *Torpedo* nAChR's apparent  $K_d$  for acetylcholine ( $10 \pm 1 \mu\text{M}$  vs. a control value of  $8 \pm 2 \mu\text{M}$ ) or to alter the maximal rate of desensitization ( $5.6 \pm 0.2 \text{ s}^{-1}$  vs. a control value of  $5.7 \pm 0.3 \text{ s}^{-1}$ ).

To further assess the agonist-potentiating abilities of cyclopropane and butane and to compare them with other anesthetics, we measured the abilities of anesthetics to increase the apparent rate of *Torpedo* nAChR desensitization induced by  $1 \mu\text{M}$  acetylcholine, an  $\text{EC}_{10}$  agonist concentration (fig. 3). We used a single-receptor preparation to more easily compare the effects of different anesthetics on the kinetics of agonist-induced desensitization without the potentially confounding effects of preparation-to-preparation variability on anesthetic sensitivity. At a concentration of 0.22 mM (0.8 MAC), isoflu-

rane increased the apparent rate of acetylcholine-induced desensitization by 56% from a control value of  $0.36 \pm 0.05 \text{ s}^{-1}$  to  $0.56 \pm 0.01 \text{ s}^{-1}$ . The apparent rate of desensitization increased with isoflurane concentration, with no evidence of saturation over the concentration range studied. By 0.88 mM, the highest concentration studied, isoflurane increased the apparent desensitization rate to  $1.4 \pm 0.2 \text{ s}^{-1}$ , a fourfold increase over the control. Ethanol and octanol also increased the apparent rate of acetylcholine-induced desensitization at pharmacologically relevant concentrations and in a concentration-dependent manner. However, at relevant concentra-



**Fig. 2.** The apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization as a function of acetylcholine concentration in the absence of anesthetic (control) and in the presence of 0.55 mM butane (3 MAC). Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 1. In the absence of anesthetic, the apparent  $K_d$  for acetylcholine was  $8 \pm 2 \mu\text{M}$ , the maximum rate of desensitization was  $5.7 \pm 0.3 \text{ s}^{-1}$ , and the Hill coefficient was  $1.2 \pm 0.2$ . The presence of 0.55 mM butane did not significantly change these values, as the apparent  $K_d$  was  $10 \pm 1 \mu\text{M}$ , the maximum rate of desensitization was  $5.6 \pm 0.2 \text{ s}^{-1}$ , and the Hill coefficient was  $1.1 \pm 0.1$ .

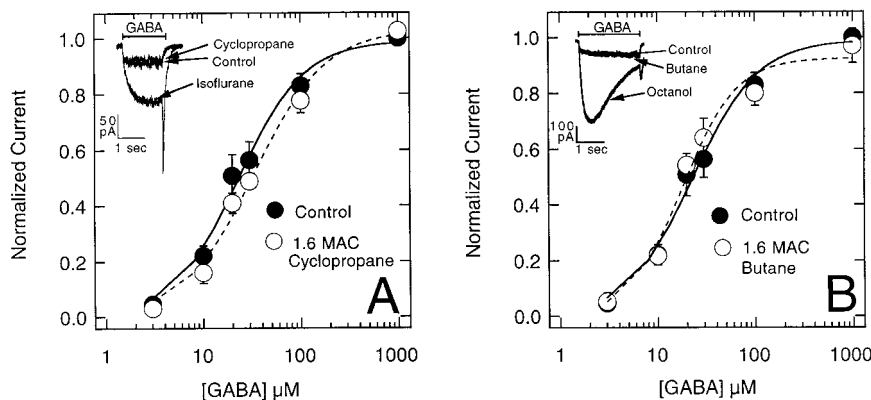


**Fig. 3.** Anesthetic concentration dependence of the apparent rate of *Torpedo* nicotinic acetylcholine receptor desensitization induced by 1  $\mu\text{M}$  acetylcholine. At all concentrations studied, isoflurane, ethanol, and octanol significantly increased the apparent rate of desensitization. Conversely, cyclopropane and butane either reduced or had no effect on the apparent rate of desensitization. The MAC of isoflurane, butane, and cyclopropane were calculated as described in Methods as 0.28, 0.16, and 0.82 mm, respectively. The MAC values of ethanol (116 mm) and octanol (37  $\mu\text{M}$ ) are derived from Fang *et al.*<sup>25</sup> \* $P < 0.05$  versus control (no anesthetic).

tions, cyclopropane and butane actually reduced the apparent rate of acetylcholine-induced desensitization rate. At higher, supraphysiologic (toxic) concentrations, the apparent desensitization rate was not significantly different from control.

#### Anesthetic Action on the $\gamma$ -Aminobutyric Acid Type A Receptor's Apparent Agonist Affinity

The inset in figure 4A shows typical current traces induced by 3  $\mu\text{M}$  GABA (an  $\text{EC}_{50}$  agonist concentration) in the absence of anesthetic (control) and in the presence of 1.6 MAC cyclopropane. Cyclopropane did not poten-



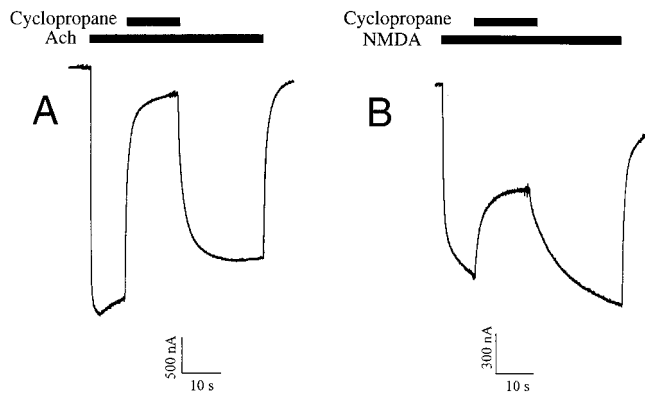
**Fig. 4.** The effect of 1.6 MAC cyclopropane and butane on  $\gamma$ -aminobutyric acid (GABA) concentration-response curves mediated by GABA type A receptors expressed in HEK 293 cells. The inset in (A) shows representative current traces induced by 3  $\mu\text{M}$  GABA and demonstrates the lack of effect of 1.6 MAC (1.3 mm) cyclopropane. For comparison, a current trace obtained in the presence of 1.6 MAC (0.44 mm) isoflurane is also shown. (A) The current response to GABA in the absence and presence of cyclopropane is also plotted. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 2. Currents were normalized to that elicited by 1 mm GABA, a saturating GABA concentration. In

the absence of anesthetic, the apparent  $K_d$  for GABA was  $23 \pm 3 \mu\text{M}$ , the maximal current was  $0.99 \pm 0.05$ , and the Hill coefficient was  $1.3 \pm 0.2$ . In the presence of cyclopropane, the apparent  $K_d$  was  $33 \pm 5 \mu\text{M}$ , the maximal current was  $1.03 \pm 0.05$ , and the Hill coefficient was  $1.2 \pm 0.2$ . The inset in (B) demonstrates the lack of effect of 1.6 MAC (0.26 mm) butane on the current induced by 3  $\mu\text{M}$  GABA. For comparison, a current trace obtained in the presence of 1.6 MAC (58  $\mu\text{M}$ ) octanol is also shown. (B) The normalized current response to GABA application in the absence and presence of butane is also plotted. Solid and dashed curves are nonlinear least squares fits of the control and anesthetic data, respectively, to equation 2. In the absence of anesthetic, the apparent  $K_d$  for GABA was  $23 \pm 3 \mu\text{M}$ , the maximal current was  $0.99 \pm 0.01$ , and the Hill coefficient was  $1.3 \pm 0.2$ . In the presence of 1.6 MAC butane, the apparent  $K_d$ , the maximal current was  $0.92 \pm 0.05$ , and the Hill coefficients were  $18 \pm 2$  and  $1.6 \pm 0.3 \mu\text{M}$ , respectively.

tiate currents induced by this low concentration of GABA. For comparison, the inset also shows the significant potentiating effect of 1.6 MAC isoflurane on currents using the same patch. The effect of 1.6 MAC cyclopropane on the GABA<sub>A</sub>R's apparent agonist  $K_d$  was determined by fitting plots of the normalized peak current as a function of GABA concentration in the presence or absence of cyclopropane to equation 2 (fig. 4A). Using this analysis, it was determined that cyclopropane did not reduce the apparent agonist  $K_d$  as the apparent  $K_d$  values for GABA in the absence and presence of cyclopropane were  $23 \pm 3$  and  $33 \pm 5 \mu\text{M}$ , respectively. The inset in figure 4B shows typical current traces induced by 3  $\mu\text{M}$  in the absence of anesthetic and in the presence of 1.6 MAC butane and 1.6 MAC octanol. Although octanol significantly potentiated currents induced by 3  $\mu\text{M}$  GABA, butane had no effect. The plot in figure 4B shows the effect of 1.6 MAC butane on the GABA concentration-response relation. In common with cyclopropane, 1.6 MAC butane failed to significantly potentiate currents induced by this low concentration of agonist or to significantly reduce the GABA<sub>A</sub>R's apparent  $K_d$  for GABA ( $18 \pm 2 \mu\text{M}$  as compared with a control value of  $23 \pm 3 \mu\text{M}$ ).

#### Inhibition of Neuronal Nicotinic Acetylcholine Receptors and N-methyl-D-Aspartate Receptors

Because our results indicated that nonhalogenated alkane anesthetics do not potentiate LGICs, we considered the possibility that they might act, instead, by inhibiting receptor function. To test this hypothesis, we determined whether cyclopropane and butane inhibit ion flux through neuronal nAChRs and NMDA receptors. Figures 5A and B demonstrate that clinically relevant concentrations of cyclopropane inhibit ion flux through neuronal

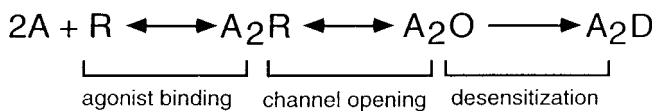


**Fig. 5.** The effect of cyclopropane on rat neuronal nicotinic acetylcholine receptor (nAChRs;  $\alpha_4$  and  $\beta_2$ ) and human *N*-methyl-D-aspartate (NMDA) receptors (NR2A and NR1b). (A) The inhibition of neuronal nAChRs caused by a pulse of 1.6 MAC cyclopropane on currents induced by 100  $\mu$ M acetylcholine. Oocytes were voltage clamped at  $-50$  mV. (B) The inhibition of NMDA receptors caused by a pulse of 0.8 MAC cyclopropane on currents induced by 100  $\mu$ M NMDA. Oocytes were voltage clamped at  $-100$  mV, and the buffer contained only  $Ba^{2+}$  as the divalent cation along with 10  $\mu$ M glycine.

nAChRs and NMDA receptors. Similarly, butane inhibits both neuronal nAChRs and NMDA receptors at clinically relevant concentrations (data not shown).

**Discussion**

The nAChR and GABA<sub>A</sub>R are the two best characterized members of the superfamily of LGICs. Functional studies indicate that the binding of two agonist molecules induces resting state receptors to undergo a rapid conformational transition to an ion-permeable open-channel state.<sup>26-28</sup> Prolonged agonist exposure induces nAChRs and GABA<sub>A</sub>Rs to undergo a slower conformational transition to one or more nonconducting desensitized state(s).<sup>26,29,30</sup> For both receptor families, the processes of agonist binding, channel gating, and desensitization may be most simply represented by the following kinetic scheme:



where A is the agonist, R is the closed resting state,  $A_2R$  is the closed preopen state,  $A_2O$  is the open channel state, and  $A_2D$  is the closed desensitized state(s). As required by this scheme and demonstrated by our data, *Torpedo* nAChR apparent desensitization rates and GABA<sub>A</sub>R currents increase with agonist concentration before reaching a plateau. An apparent agonist  $K_d$  may then be defined from such agonist concentration-response curves as the agonist concentration required to

induce a half-maximal response. In the current study we used two different methods for determining the apparent agonist  $K_d$ . For the *Torpedo* nAChR, we used a nonflux fluorescence assay that determines the apparent agonist  $K_d$  from the apparent rate of acetylcholine-induced desensitization measured over a wide range of acetylcholine concentrations. This technique (rather than ion flux techniques) was chosen to measure the apparent agonist  $K_d$  because many anesthetics not only increase the *Torpedo* nAChR's apparent agonist affinity, but also inhibit ion flux through it.<sup>8,31,32</sup> Such inhibition can mask anesthetic-induced agonist potentiation when electrophysiologic or radiotracer flux techniques are used.<sup>9</sup> For the GABA<sub>A</sub>R (which is not as potently blocked by anesthetics), we used a standard electrophysiologic approach and calculated the apparent agonist  $K_d$  from the GABA concentration dependence of the measured peak current. We chose cyclopropane and butane as representative nonhalogenated alkane anesthetics because human potency data are available for both of these anesthetics and because concentrations in excess of those required to induce anesthesia may be readily achieved.

We have previously demonstrated that in the *Torpedo* nAChR, isoflurane and normal alcohols increase the apparent rate of desensitization induced by low concentrations of agonist.<sup>33</sup> Subsequent studies using a wide range of agonist concentrations demonstrated that these anesthetics induce a leftward shift in acetylcholine-response curves for desensitization.<sup>8,9</sup> This is indicative of a reduction in the *Torpedo* nAChR's apparent agonist  $K_d$ . In these studies, we found that anesthetics reduced the apparent agonist  $K_d$  by approximately half at clinical anesthetic concentrations. Furthermore, we observed that the apparent agonist  $K_d$  decreased logarithmically with anesthetic concentration without clear evidence of saturation even at relatively high concentrations. Consequently, high concentrations of isoflurane and normal alcohols can reduce the *Torpedo* nAChR's apparent agonist  $K_d$  by 10-100-fold. One of the principle results of this study is that even at high concentrations that are more than sufficient to induce anesthesia, cyclopropane and butane fail to potentiate the effects of low agonist concentrations or to reduce the apparent agonist  $K_d$  of the *Torpedo* nAChR.

At clinically relevant concentrations, a wide range of general anesthetics also potentiate the actions of low GABA concentrations on GABA<sub>A</sub>Rs.<sup>11</sup> In common with the *Torpedo* nAChR, studies using a range of agonist concentrations indicate that this potentiation reflects a reduction in the GABA<sub>A</sub>R's apparent agonist  $K_d$ .<sup>3,34</sup> Because GABA is a major inhibitory neurotransmitter in the central nervous system, it is thought that the central nervous system-depressant effects of general anesthetics may result from their abilities to potentiate GABA's actions on GABA<sub>A</sub>Rs. In light of our data demonstrating

that *Torpedo* nAChRs are unaffected by clinically relevant concentrations of cyclopropane and butane, and given the similarity in structure and function between the nAChR and GABA<sub>A</sub>R, we hypothesized that GABA<sub>A</sub>R might also be insensitive to clinically relevant concentrations of these cyclopropane and butane. To test this hypothesis, we studied the effect of 1.6 MAC concentrations of cyclopropane and butane on the apparent agonist affinity of the GABA<sub>A</sub>R. Figures 4A and B demonstrate that at these concentrations, both cyclopropane and butane fail to reduce the GABA<sub>A</sub>R's apparent agonist K<sub>d</sub>. Thus, not only are *Torpedo* nAChRs and GABA<sub>A</sub>R both sensitive to agonist potentiation by isoflurane and normal alcohols, they are both relatively insensitive to agonist potentiation by cyclopropane and butane. This correlation between anesthetic action on the *Torpedo* nAChR and GABA<sub>A</sub>R suggests that the underlying molecular mechanism(s) responsible for agonist potentiation in both superfamily members is the same. Furthermore, the inability of cyclopropane and butane to reduce the apparent agonist K<sub>d</sub> of the GABA<sub>A</sub>R at a concentration that induces anesthesia strongly suggests that other molecular mechanisms likely account for their *in vivo* anesthetic activities. Although we have not yet studied the activities of nonhalogenated anesthetics on other superfamily members (5-hydroxytryptamine<sub>3</sub> or glycine receptors), the observation that they do not potentiate agonist actions on either the nAChR or the GABA<sub>A</sub>R suggests that they also fail to potentiate these LGICs. As these anesthetics potently inhibit neuronal nAChRs and NMDA receptors, these ion channels must be considered to be potentially relevant targets of nonhalogenated alkane anesthetics.

A recent study by de Sousa *et al.*<sup>35</sup> identified xenon as another anesthetic that does not potentiate the GABA<sub>A</sub>R at a clinically relevant concentration (1.1 MAC). In an accompanying editorial, Harrison<sup>36</sup> suggested that xenon may fail to potentiate agonist action because it is unable to participate in hydrogen-bonding interactions with the receptor. Such interactions, which are possible for ether, alcohol, and nearly all halogenated alkane anesthetics, have been suggested to augment binding affinity to a model protein by an order of magnitude.<sup>37</sup> Our data demonstrating that at 1.6 MAC, cyclopropane and butane have no effect on the GABA<sub>A</sub>R's apparent K<sub>d</sub> is generally consistent with Harrison's suggestion as these two anesthetics are also incapable of forming hydrogen bonds. Alternatively, Cafiso *et al.* and Pohorille *et al.* have suggested that an anesthetic's dipole moment mediates its interactions with membrane targets.<sup>38-41</sup> Because nonhalogenated alkanes (and xenon) are among the very few anesthetics that do not possess a permanent dipole moment, we cannot rule out a critical role for the dipole moment in modulating anesthetic action on the *Torpedo* nAChR and GABA<sub>A</sub>R.

In summary, our studies show that, at concentrations

that are sufficient to induce anesthesia, neither cyclopropane nor butane significantly increases the apparent agonist affinities of two representative members of the superfamily of LGICs, the *Torpedo* nAChR and the GABA<sub>A</sub>R. Such findings strongly suggest that agonist potentiation of LGICs does not account for the anesthetic activities of nonhalogenated alkane anesthetics.

Dns-C<sub>6</sub>-Cho was synthesized by S. Shaikat Husain, D.Phil. (Research Associate in Anesthesia, Massachusetts General Hospital, Boston, Massachusetts); GABA<sub>A</sub> receptor cDNAs were supplied by Paul J. Whiting, Ph.D. (Research Scientist, Merck Sharp & Dohme Research Labs, Essex, United Kingdom); neuronal nicotinic receptor subunit cDNAs were gifts from James Boulter, Ph.D. (Associate Professor of Psychiatry and Biobehavioral Sciences, Salk Institute, San Diego, California); NMDA receptor subunit cDNAs were gifts from Steven Triestman, Ph.D. (Professor of Pharmacology, University of Massachusetts Medical Center, Worcester, Massachusetts).

## References

1. Stroud RM, McCarthy MP, Shuster M: Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry* 1990; 29:11009-23
2. Changeux JP: The TiPS lecture. The nicotinic acetylcholine receptor: An allosteric protein prototype of ligand-gated ion channels. *Trends Pharmacol Sci* 1990; 11:485-92
3. Li X, Pearce RA: Effects of halothane on GABA(A) receptor kinetics: evidence for slowed agonist unbinding. *J Neurosci* 2000; 20:899-907
4. Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL: Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 1997; 389:385-9
5. Violet JM, Downie DL, Nakisa RC, Lieb WR, Franks NP: Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anesthetics. *ANESTHESIOLOGY* 1997; 86:866-74
6. Yamakura T, Harris RA: Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels: Comparison with isoflurane and ethanol. *ANESTHESIOLOGY* 2000; 93:1095-101
7. Liu Y, Dilger JP, Vidal AM: Effects of alcohols and volatile anesthetics on the activation of nicotinic acetylcholine receptor channels. *Mol Pharmacol* 1994; 45:1235-41
8. Raines DE, Zachariah VT: Isoflurane increases the apparent agonist affinity of the nicotinic acetylcholine receptor. *ANESTHESIOLOGY* 1999; 90:135-46
9. Raines DE, Zachariah VT: The alkyl chain dependence of the effect of normal alcohols on agonist-induced nicotinic acetylcholine receptor desensitization kinetics. *ANESTHESIOLOGY* 1999; 91:222-30
10. Raines DE, Zachariah VT: Isoflurane increases the apparent agonist affinity of the nicotinic acetylcholine receptor by reducing the microscopic dissociation constant. *ANESTHESIOLOGY* 2000; 92:755-85
11. Zimmerman SA, Jones MV, Harrison NL: Potentiation of gamma-aminobutyric acidA receptor Cl<sup>-</sup> current correlates with *in vivo* anesthetic potency. *J Pharmacol Exp Ther* 1994; 270:987-91
12. Zhou Q, Verdoorn TA, Lovinger DM: Alcohols potentiate the function of 5-HT<sub>3</sub> receptor-channels on NCB-20 neuroblastoma cells by favouring and stabilizing the open channel state. *J Physiol (Lond)* 1998; 507:335-52
13. Jenkins A, Franks NP, Lieb WR: Actions of general anaesthetics on 5-HT<sub>3</sub> receptors in N1E-115 neuroblastoma cells. *Br J Pharmacol* 1996; 117:1507-15
14. Machu TK, Harris RA: Alcohols and anesthetics enhance the function of 5-hydroxytryptamine<sub>3</sub> receptors expressed in *Xenopus laevis* oocytes. *J Pharmacol Exp Ther* 1994; 271:898-905
15. Downie DL, Hall AC, Lieb WR, Franks NP: Effects of inhalational general anaesthetics on native glycine receptors in rat medullary neurones and recombinant glycine receptors in *Xenopus* oocytes. *Br J Pharmacol* 1996; 118:493-502
16. Waksman G, Fournie ZMC, Roques B: Synthesis of fluorescent acylcholines with agonist properties: Pharmacological activity on *Electrophorus* electroplaque and interaction *in-vitro* with *Torpedo* receptor-rich membrane fragments. *Febs Lett* 1976; 67:335-42
17. Braswell LM, Miller KW, Sauter JF: Pressure reversal of the action of octanol on postsynaptic membranes from *Torpedo*. *Br J Pharmacol* 1984; 83:305-11
18. Raines DE, Krishnan NS: Transient low-affinity agonist binding to *Torpedo* postsynaptic membranes resolved by using sequential mixing stopped-flow fluorescence spectroscopy. *Biochemistry* 1998; 37:956-64
19. Chen C, Okayama H: High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 1987; 7:2745-52
20. Jurman ME, Boland LM, Liu Y, Yellen G: Visual identification of individual transfected cells for electrophysiology using antibody-coated beads. *Biotechniques* 1994; 17:876-81

21. Forman SA: A hydrophobic photolabel inhibits nicotinic acetylcholine receptors via open-channel block following a slow step. *Biochemistry* 1999; 38:14559-64
22. Forman SA: Homologous mutations on different subunits cause unequal but additive effects on n-alcohol block in the nicotinic receptor pore. *Biophys J* 1997; 72:2170-9
23. Firestone LL, Miller JC, Miller KW: Table of physical and pharmacological properties of anesthetics, Molecular and Cellular Mechanisms of Anesthetics. Edited by Roth SH, Miller KW. New York, Plenum Press, 1986, pp 455-70
24. Franks NP, Lieb WR: Selective actions of volatile general anaesthetics at molecular and cellular levels. *Br J Anaesth* 1993; 71:65-76
25. Fang Z, Ionescu P, Chortkoff BS, Kandel L, Sonner J, Laster MJ, Eger EI 2nd: Anesthetic potencies of n-alkanols: Results of additivity and solubility studies suggest a mechanism of action similar to that for conventional inhaled anesthetics. *Anesth Analg* 1997; 84:1042-8
26. Neubig RR, Boyd ND, Cohen JB: Conformations of Torpedo acetylcholine receptor associated with ion transport and desensitization. *Biochemistry* 1982; 21:3460-7
27. Sine SM, Steinbach JH: Activation of acetylcholine receptors on clonal mammalian BC3H-1 cells by high concentrations of agonist. *J Physiol (Lond)* 1987; 385:325-59
28. Jones MV, Westbrook GL: Desensitized states prolong GABAA channel responses to brief agonist pulses. *Neuron* 1995; 15:181-91
29. Boyd ND, Cohen JB: Kinetics of binding of [3H]acetylcholine and [3H]carbamoylcholine to Torpedo postsynaptic membranes: Slow conformational transitions of the cholinergic receptor. *Biochemistry* 1980; 19:5344-53
30. Jones MV, Westbrook GL: The impact of receptor desensitization on fast synaptic transmission. *Trends Neurosci* 1996; 19:96-101
31. Forman SA, Miller KW, Yellen G: A discrete site for general anesthetics on a postsynaptic receptor. *Mol Pharmacol* 1995; 48:574-81
32. Forman SA, Raines DE: Nonanesthetic volatile drugs obey the Meyer-Overton correlation in two molecular protein site models. *ANESTHESIOLOGY* 1998; 88:1535-48
33. Raines DE, Rankin SE, Miller KW: General anesthetics modify the kinetics of nicotinic acetylcholine receptor desensitization at clinically relevant concentrations. *ANESTHESIOLOGY* 1995; 82:276-87
34. Quinlan JJ, Firestone S, Firestone LL: Isoflurane's enhancement of chloride flux through rat brain gamma-aminobutyric acid type A receptors is stereoselective. *ANESTHESIOLOGY* 1995; 83:611-5
35. de Sousa SL, Dickinson R, Lieb WR, Franks NP: Contrasting synaptic actions of the inhalational general anesthetics isoflurane and xenon. *ANESTHESIOLOGY* 2000; 92:1055-66
36. Harrison NL: Ion channels take center stage: Twin spotlights on two anesthetic targets. *ANESTHESIOLOGY* 2000; 92:936-8
37. Eckenhoff RG, Johansson JS: Molecular interactions between inhaled anesthetics and proteins. *Pharmacol Rev* 1997; 49:343-67
38. Qin Z, Szabo G, Cafiso DS: Anesthetics reduce the magnitude of the membrane dipole potential: Measurements in lipid vesicles using voltage-sensitive spin probes. *Biochemistry* 1995; 34:5536-43
39. North C, Cafiso DS: Contrasting membrane localization and behavior of halogenated cyclobutanes that follow or violate the Meyer-Overton hypothesis of general anesthetic potency. *Biophys J* 1997; 72:1754-61
40. Cafiso DS: Dipole potentials and spontaneous curvature: Membrane properties that could mediate anesthesia. *Toxicol Lett* 1998; 100-101:431-9
41. Pohorille A, Wilson MA, New MH, Chipot C: Concentrations of anesthetics across the water-membrane interface; the Meyer-Overton hypothesis revisited. *Toxicol Lett* 1998; 100-101:421-30