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Nonhalogenated Alkanes Cyclopropane and Butane Affect Neurotransmitter-gated Ion Channel and G-protein–coupled Receptors

Differential Actions on $GABA_A$ and Glycine Receptors

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Background: **Anesthetic mechanisms of nonhalogenated alkanes cyclopropane and butane are not understood. This study was designed to look at which neurotransmitter receptors are possible targets for these anesthetics.**

Methods: **Effects of cyclopropane and butane on eight recombinant receptors expressed in *Xenopus* oocytes were examined electrophysiologically. To address molecular mechanisms of interaction with glycine and γ-aminobutyric acid type A ($GABA_A$) receptors, cyclopropane was further tested on α_1(S267C) glycine receptor and α_2(S270X)β_1 $GABA_A$ receptors that were mutated to amino acids with larger side chains.**

Results: **Cyclopropane (1, 2, and 5 minimum alveolar concentration [MAC]) potentiated glycine responses by 39, 62, and 161%, respectively, and butane (1 MAC) potentiated by 64% with an increase in apparent affinity for glycine, but yielded barely detectable potentiation of $GABA_A$ receptors. The efficacy of cyclopropane for glycine receptors was less than isoflurane and halothane. The potentiation by cyclopropane was eliminated for the α_1(S267C) glycine receptor. Mutant $GABA_A$ receptors in which the corresponding amino acid was substituted with larger amino acids did not produce significant potentiation. Cyclopropane and butane inhibited nicotinic acetylcholine and *N*-methyl-D-aspartate receptors, potentiated G-protein–coupled inwardly rectifying potassium channels, and did not change 5-hydroxytryptamine$_{3A}$ or muscarinic$_1$ receptor function. Only cyclopropane markedly inhibited α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors.**

Conclusions: **Glycine, nicotinic acetylcholine, and *N*-methyl-D-aspartate receptors are sensitive to nonhalogenated alkanes, and the authors propose that glycine and *N*-methyl-D-aspartate receptors are good candidates for anesthetic immobility. The authors also suggest that the distinct effects on glycine and $GABA_A$ receptors are not due to the small volumes of these anesthetics.**

NEUROTRANSMITTER-GATED ion channels, such as γ-aminobutyric acid type A ($GABA_A$), glycine, neuronal nicotinic acetylcholine (nACh), 5-hydroxytryptamine$_3$ (5-HT$_3$), and/or *N*-methyl-D-aspartate (NMDA) receptors, are known to be sensitive to most anesthetics, indicating that these channels are plausible targets for anesthetics.[1–3] A wide variety of structurally diverse general anesthetics, including inhaled and intravenous anesthetics, enhance $GABA_A$ receptor function. In addition to the neurotransmitter-gated ion channels, some anesthetics are also known to affect G-protein–coupled receptors, such as muscarinic$_1$ (M_1) receptors[4] and G-protein–coupled inwardly rectifying potassium (GIRK) channels.[5] These molecules may account for some clinical aspects of anesthesia by anesthetics and ethanol, including analgesia, amnesia, and sedation.[6–8]

A recent report showed that two alkane anesthetics, cyclopropane and butane, failed to increase agonist affinity for the $GABA_A$ and Torpedo nACh receptors and that they suppressed the functions of nACh and NMDA receptors.[9] This suggests a spectrum of action that is different from halogenated alkane anesthetics, but effects of cyclopropane and butane on other receptors have not been examined. In this study, we examine other targets that could contribute to anesthesia (immobility) produced by nonhalogenated alkanes.

We tested the effects of nonhalogenated alkane anesthetics on recombinant neurotransmitter-gated ion channels ($\alpha_1\beta_2\gamma_{2S}$ and $\alpha_2\beta_1$ $GABA_A$, α_1 glycine, NR1/NR2A NMDA, GluR1/GluR2 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid [AMPA], $\alpha_4\beta_2$ neuronal nACh, 5HT$_{3A}$), and G-protein–coupled receptors (M_1) and ion channels (GIRK1/2) expressed in *Xenopus* oocytes. The subunit compositions of the recombinant receptors were chosen on the basis of the predominance of subunit distribution in the central nervous system (CNS).

In this study, we found that cyclopropane and butane at clinical concentrations moderately enhance the glycine receptors, whereas $GABA_A$ receptors are insensitive. To elucidate the mechanism underlying the differential effects on two inhibitory receptors, we hypothesized that a difference in the volume of binding cavity for the anesthetics is the determinant of anesthetic modulation. We extended mutational study for glycine and $GABA_A$ receptors as to a position where anesthetics and ethanol have been proven to interact with both receptors and knew that the difference in the volume cannot account for the difference in sensitivity between the glycine and the $GABA_A$ receptors. We also discuss which signaling systems might be mediators of immobility by these anesthetics.

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Materials and Methods

These studies were approved by the Animal Care and Use Committee of the University of Texas (Austin, Texas).

cDNA and cRNA Preparations

The cDNAs encoding the following receptor subunits were used for nuclear injections: human wild-type α_1 or α_1(S267C) glycine (subcloned in pCIS2 vector)[10]; wild-type human α_1 (subcloned in pBK-CMV N/B-200 vector); α_2, β_1, γ_{2S}, and α_2 mutants (S270X) (subcloned in pCIS2 vector) and β_2 (subcloned in pCDM8 vector) $GABA_A$[11]; human NR1 and NR2A NMDA (subcloned in pcDNA Amp vector)[12]; and human 5-HT_{3A} (subcloned in pBK-CMV N/B-200 vector).[13] The cDNAs of rat GluR1 and GluR2 AMPA receptor subunits (subcloned in pBluescript SK^- vector)[14]; the cDNAs of rat α_4 and β_2 nACh receptor subunits (subcloned in pSP64 and pSP65 vectors, respectively)[15]; the cDNAs of rat GIRK1[16] and mouse GIRK2[17] (subcloned in pBluescript II KS^- vector); and the cDNA of rat M_1 receptor (subcloned in pGEM vector)[18] were used for cRNA synthesis. *In vivo* transcripts were prepared using the mCAP Capping Kit (Stratagene, La Jolla, CA).

Expression of the Receptors in Oocytes

Isolation of *Xenopus laevis* oocytes was conducted as described previously.[19] Isolated oocytes were placed in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.91 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) adjusted to pH 7.5. Single cDNAs or combinations of the cDNAs encoding the following receptor subunits were injected into the animal poles of oocytes by a blind method[20]: the α_1 or α_1(S267C) glycine receptor subunit cDNA (1 ng/30 nl); the α_1, β_2, and γ_{2S} $GABA_A$ receptor subunits cDNAs (2 ng/30 nl in a 1:1:2 molar ratio); the α_2 (wild-type or mutants) and β_1 $GABA_A$ receptor subunits cDNAs (1.5 ng/30 nl in a 1:1 molar ratio); the NR1 and NR2A NMDA receptor subunits cDNAs (1.5 ng/30 nl in a 1:1 molar ratio); the 5-HT_{3A} receptor subunit cDNA (1.5 ng/30 nl). The GluR1 and GluR2 AMPA receptor subunits cRNAs, the α_4 and β_2 nACh receptor subunits cRNAs, the GIRK1 and GIRK2 receptor subunits cRNAs (30 ng/30 nl in a 1:1 molar ratio), or M_1 receptor subunit cRNA (30 ng/30 nl) were injected into cytoplasm of oocytes. The injected oocytes were singly placed in Corning cell wells (Corning Glass Works, Corning, NY) containing incubation medium (sterile MBS supplemented with 10 mg/l streptomycin, 100,000 U/l penicillin, 50 mg/l gentamycin, 90 mg/l theophylline, and 220 mg/l pyruvate) and incubated at 15–19°C. One to 4 days after injection, the oocytes were used in electrophysiological recording.[19]

Electrophysiological Recording

Oocytes expressing $GABA_A$, glycine, AMPA, or M_1 receptors were placed in a rectangular chamber (~100-μl volume) and perfused (2 ml/min) with MBS. Oocytes expressing NMDA receptors were perfused with Ba^{2+} Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES adjusted to pH7.4) to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- currents, and oocytes expressing nACh receptors were perfused with Ba^{2+} Ringer's solution containing 1 μM atropine sulfate. For the 5-HT_{3A} receptors, oocytes were perfused with low-Ca^{2+} Ringer's solution (115 mM NaCl, 2.5 mM KCl, 0.18 mM $CaCl_2$, and 10 mM HEPES adjusted to pH7.4) to decrease inhibitory effect of calcium ion. The animal poles of oocytes were impaled with two glass electrodes (0.5–10 Ω) filled with 3 M KCl, and the oocytes were voltage clamped at −70 mV by using a Warner Instruments model OC-752B oocyte clamp (Hamden, CT). Glycine, GABA, kainic acid (for AMPA receptors), or ACh (for M_1 receptor) dissolved in MBS was applied to the oocytes for 20 or 30 s to reach a maximal response. Likewise, L-glutamate plus 10 μM glycine (for NMDA receptors) or ACh (for nACh receptor) dissolved in Ba^{2+} Ringer's solution was applied to the oocytes for 20 s, and 5-HT in low-Ca^{2+} Ringer's solution was applied for 30 s. To study the effects on both wild-type and mutant $GABA_A$ or glycine receptors, the experiments were performed at EC_5 of agonist (concentrations that produced 5% of the maximal currents produced by 1 mM of glycine or GABA). To study the effects on the NMDA, AMPA, nACh, M_1, or 5-HT_{3A} receptors, the experiments were performed at the half-maximal effective concentration (EC_{50}) of agonists. Based on the concentration–response relation from our previous work,[4,21,22] which was performed under the same conditions as the current study (receptor: EC_{50}, Hill coefficient; α_1 glycine: 206 ± 18 μM, 2.1 ± 0.1; $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$: 94 ± 3 μM, 1.2 ± 0.04; NR1/NR2A: 2.1 ± 0.3 μM, 1.3 ± 0.1; GluR1/GluR2: 91 ± 4 μM, 1.4 ± 0.1; 5-HT_{3A}: 1.8 ± 0.2 μM, 2.1 ± 0.1; $\alpha_4\beta_2$ nACh: 60 ± 3 μM, 0.9 ± 0.1; M_1 ACh: 0.9 μM, 1.1), we used agonist concentrations to obtain EC_5 or EC_{50} for each receptor as follows: 30–60 μM glycine for wild-type α_1 and α_1(S267C) glycine receptors, 10–30 μM GABA for $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$, and 1–2 μM GABA for $\alpha_2\beta_1$ $GABA_A$ receptors; 60 μM ACh for nACh receptors; 1.5–2 μM 5-HT for 5-HT_{3A} receptors; 2–3 μM L-glutamate plus 10 μM glycine for NMDA receptors; 100 μM kainic acid for AMPA receptors; and 1 μM ACh for M_1 receptors. To obtain a control response, the agonists were repeatedly applied until a consistent response was observed. A 5- to 20-min washout period was allowed between drug applications.

Each solution (20 ml) in a closed vial was bubbled with 100% cyclopropane or butane for at least 10 min at room temperature to obtain a saturated solution. The saturated

solution was pumped into the rectangular chamber *via* a roller pump (ColeParmer Instrument Co., Chicago, IL) through 18-gauge polyethylene tubing. The concentrations of cyclopropane and butane in the chamber were quantified by gas chromatography, and the values were 0.46 ± 0.03 atm (n = 3) and 0.29 ± 0.01 atm (n = 3), respectively. These concentrations correspond to 5 minimum alveolar concentration (MAC) of cyclopropane[23] and 1 MAC of butane.[24] To test the effects of 1 and 2 MAC of cyclopropane, the saturated solutions were diluted with cyclopropane-free solution immediately before application. Cyclopropane or butane was preapplied for 1 min before being coapplied with agonists, unless specified. Effects of anesthetics were expressed as the fraction of control responses, which were calculated by averaging the control responses before and after application of anesthetics. To rule out the involvement of hypoxia, the effect of a perfusion solution bubbled with 100% nitrogen was evaluated in a previous study; replacement of oxygen by nitrogen did not alter the function of several ligand-gated ion channels.[21]

To address the mechanism of cyclopropane action on the α_1 glycine receptor, we examined the glycine concentration–response relation in the presence or absence of cyclopropane, butane, or isoflurane. For other receptors, such as nACh, NMDA, and 5-HT_{3A}, we examined the effects of 5 MAC cyclopropane on the maximal response to agonists. According to the concentration–response relations obtained from our previous work,[21,22] 1 mM ACh, 100 μM L-glutamate plus 10 μM glycine, or 30 μM 5-HT were applied to obtain maximal responses.

Experiments with the GIRK1/2 channels were performed with a high potassium (hK) solution containing 2 mM NaCl, 96 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 5 mM HEPES adjusted to pH 7.5. Initially, oocytes expressing GIRK1/2 were bathed in MBS and changed in hK solution. Because basal current obtained in physiologic buffer (MBS) is small, hK solution was used to reverse the driving force of the channel and provide a large inward current, as reported previously.[5] After stable responses were established, the anesthetics were applied in the hK solution for 2 min before returning to the anesthetic-free hK solution. Effect of the anesthetics was expressed as the fraction of hK responses. All experiments were performed at room temperature (23°C).

Mutational Studies on $GABA_A$ and Glycine Receptors

Our previous studies showed that serine at position 267 located between transmembrane domains 2 and 3 of the glycine α_1 subunit and the corresponding amino acids of $GABA_A$ α and β subunits are critical for the positive modulation of the receptor functions by several anesthetics and ethanol.[25–27] Accordingly, we tested the effect of cyclopropane on the homomeric α_1(S267C) mutant glycine receptor in which serine at position 267 is substituted with cysteine. Also, we studied effects of cyclopropane on α_2 mutant $GABA_A$ receptors in which the corresponding sS270 was substituted with amino acids with larger volume of the side chain, *i.e.*, isoleucine (S270I), asparagine (S270N), threonine (S270T), tyrosine (S270Y), and tryptophan (S270W). The mutated glycine α_1(S267C) subunit and $GABA_A$ α_2(S270X) subunits were constructed by the method of site-directed mutagenesis as described previously.[28] The glycine α_1(S267C) subunit and $GABA_A$ α_2(S270X) subunit were confirmed by DNA sequencing.

Animal and Chemicals

Xenopus laevis female frogs were purchased from Xenopus Express (Homosassa, FL). Cyclopropane, butane, glycine, L-glutamate, kainic acid, acetylcholine chloride, and 5-hydroxytryptamine hydrochloride were obtained from Sigma Aldrich (St. Louis, MO). GABA was obtained from Research Biochemical International (Natick, MA).

Statistical Analysis

Data are represented as mean ± SEM. Statistical analysis was performed by one-way analysis of variance for multiple comparisons and unpaired Student *t* test for comparisons between two groups. Differences were considered as statistically significant at a *P* value less than 0.05. The values of the EC_{50} and the Hill coefficient were calculated by nonlinear regression using GraphPad Prism software version 3.0 (GraphPad Inc., San Diego, CA). Concentration–response data for glycine were fitted to the equation)

$$I/I_{max} = 1/[1 + (EC_{50}/A)^n],$$

where I represents the current, I_{max} represents the maximal current, A represents the agonist (glycine) concentration, and n represents the Hill coefficient. Molecular volumes of anesthetics and side chain of amino acid were calculated with Spartan 5.0 software (Wavefunction Inc., San Diego, CA).

Results

Differential Effects of Cyclopropane between $GABA_A$ and Glycine Receptors

In oocytes expressing $GABA_A$ or glycine receptors, inward chloride currents were observed in response to the applications of agonists, and effects of anesthetics were studied by coapplication with agonists (fig. 1). For $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, cyclopropane showed little effect at 1 or 2 MAC (fig. 2A). Even at 5 MAC, it only modestly potentiated the receptor (18 ± 2%). To test subunit specificity, we also used the $\alpha_1\beta_2$ $GABA_A$ receptor and cyclopropane potentiated the $\alpha_2\beta_1$ $GABA_A$ receptor to a similar extent as the $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor

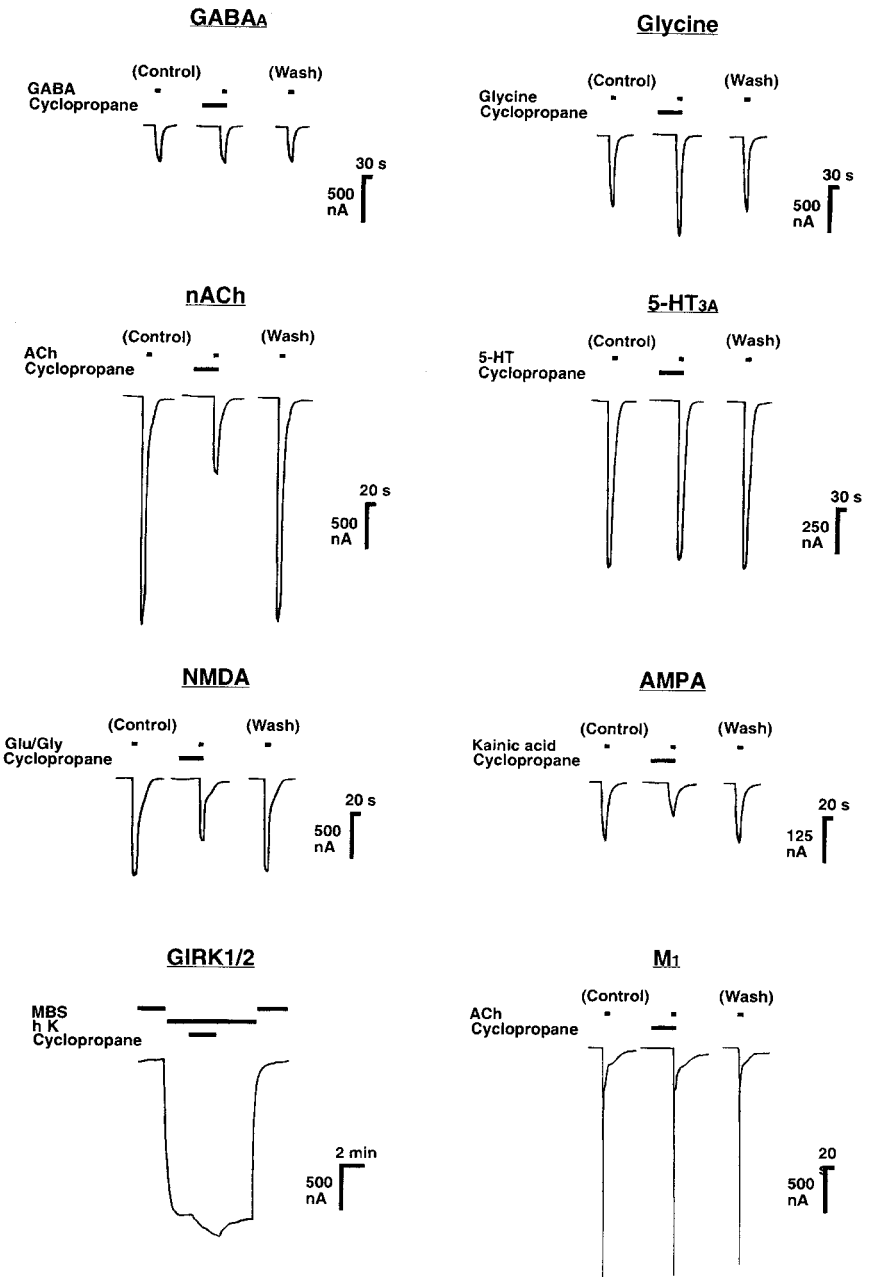

Fig. 1. Representative tracings of the currents of recombinant receptors expressed in *Xenopus* oocytes. Oocytes expressing the receptors were first exposed to the agonists (Control), then 1 minimum alveolar concentration (MAC) of cyclopropane was preapplied for 1 min before being coapplied with agonists followed by 5–20 min of washout period (Wash). Subunit compositions of the recombinant receptors were chosen on the basis of the predominance of subunit distribution in the central nervous system. Bars represent duration of application.

(16% ± 2%). Thus, it appears that the γ subunit does not influence cyclopropane action on $GABA_A$ receptors. On the other hand, cyclopropane at 1, 2, and 5 MAC significantly potentiated the current responses of the α_1 glycine receptors by 39, 62, and 161%, respectively. Butane was tested at 1 MAC, which is the highest concentration possible in our system. Butane significantly potentiated the $\alpha_1\beta_2$ $GABA_A$ receptors by 13% and α_1 glycine receptors by 64% (fig. 2B). We compared cyclopropane with isoflurane and halothane actions on glycine receptors. Isoflurane at 1, 2, and 5 MAC potentiated the current responses of the α_1 glycine receptors by 101, 209, and 484%, respectively. Similarly, halothane potentiated by 139, 253, and 534% (1, 2, and 5 MAC, respectively). Isoflurane and halothane seem to be roughly three times more effective than cyclopropane (fig. 3).

Cyclopropane and butane shifted the glycine concentration–response curve leftward as was seen for isoflurane (fig. 4). In the experiments with cyclopropane, nonlinear regression analysis of the concentration response curves yielded the glycine EC_{50} value of 118 ± 6 μM for control and 69 ± 5 μM for cyclopropane ($P < 0.05$), and the Hill coefficients for control and cyclopropane were 2.0 and 2.3, respectively. In the experiments with butane, the glycine EC_{50} value of 142 ± 6 μM for control and 109 ± 4 μM for butane ($P < 0.05$) and the Hill coefficients both for control and for cyclopropane were 1.6. In the experiments with isoflurane, we ob-

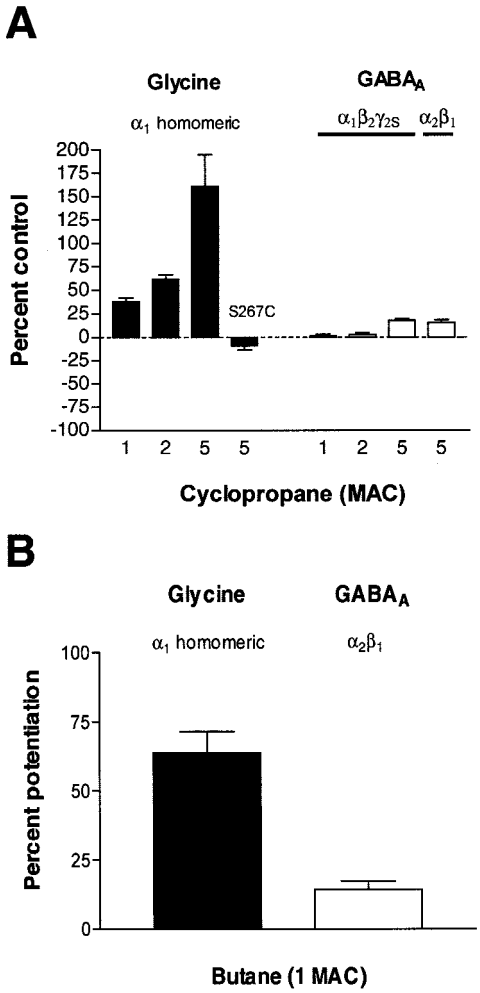

Fig. 2. Effects of nonhalogenated alkanes cyclopropane and butane on recombinant $GABA_A$ and glycine receptors. (*A*) Cyclopropane (1, 2, and 5 minimum alveolar concentration [MAC]) significantly enhanced the current responses of α_1 glycine receptors to an EC_5 of agonists. On α_1(S267C) mutant receptor, the enhancing effect of cyclopropane (5 MAC) was completely eliminated. On the other hand, $GABA_A$ receptors required 5 MAC of cyclopropane for a small, but significant, potentiation. Cyclopropane showed similar effects on $\alpha_2\beta_1$ $GABA_A$ receptors. (*B*) Butane (1 MAC) significantly enhanced the α_1 glycine and the $\alpha_2\beta_1$ $GABA_A$ receptors with much higher potentiation of the glycine receptors than the $GABA_A$ receptors. Error bars represent SEM; n = 6–13 oocytes.

tained a glycine EC_{50} value of 126 ± 6 μM for control and 31 ± 8 μM for isoflurane ($P < 0.05$), and the Hill coefficients for control and isoflurane were 1.8 and 1.6, respectively. These results suggest that both cyclopropane and butane increase the apparent affinity for glycine as do other volatile anesthetics. The enhancing effect of cyclopropane was completely abolished in the glycine α_1(S267C) receptor (−8% ± 4%; fig. 2A), indicating that cyclopropane interacts with the putative binding pocket composed of serine at position 267 as do ethanol and anesthetics including isoflurane and enflurane.[25–27]

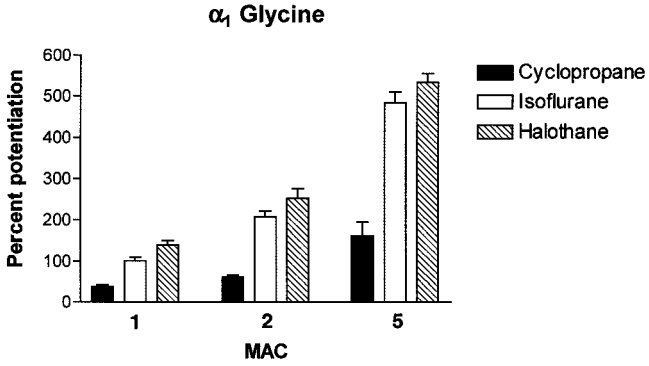

Fig. 3. Comparison of enhancing effects of cyclopropane, isoflurane, and halothane on recombinant α_1 glycine receptors. Cyclopropane, isoflurane, and halothane (1, 2, and 5 minimum alveolar concentration [MAC]) significantly enhanced the current responses of the glycine receptors to an EC_5 of glycine. Cyclopropane had roughly two to three times less effect than isoflurane and halothane. Error bars represent SEM; n = 8–13 oocytes.

Effects on Other Neurotransmitter-gated and G-protein–coupled Receptors

For the experiments testing the nACh, glutamate, or 5-HT_{3A} receptors, oocytes expressing the $\alpha_4\beta_2$ nACh, NR1/NR2A NMDA, GluR1/GluR2 AMPA, or 5-HT_{3A} receptors represented inward cation currents in response to agonists (fig. 1). Cyclopropane inhibited the nACh receptor in a concentration-dependent manner (fig. 5A). One MAC cyclopropane markedly suppressed the nACh receptor function by 70 ± 4%. Similarly, cyclopropane concentration-dependently inhibited the NMDA and AMPA glutamate receptors. These receptors were significantly inhibited by 1 MAC cyclopropane (NMDA, −29 ± 5%; AMPA, −51 ± 5%). The 5-HT_{3A} receptors were less sensitive than the nACh and glutamate receptors and slightly suppressed by 2 MAC. At concentrations of agonists that produce maximal responses (EC_{100}), the inhibitory effect of 5 MAC cyclopropane on the 5-HT_{3A} receptors was not changed (EC_{50}, 23 ± 4%; EC_{100}, 17 ± 4%). On the other hand, the inhibitory effects of cyclopropane on the NMDA and the nACh receptors were decreased at agonist EC_{100} (NMDA, 5 MAC: EC_{50}, −66 ± 4%; EC_{100}, −33 ± 3%; nACh, 1 MAC: EC_{50}, −70 ± 1%; EC_{100}, −54 ± 3%). Cyclopropane enhanced the function of the GIRK1/2 channels (fig. 5A). One MAC cyclopropane slightly but significantly increased potassium current by 9 ± 1%. *Xenopus* oocyte expression system is also well characterized for the study of G-protein–coupled receptors. Activation of M_1 receptors expressed in oocytes results in activation of phospholipase C, mobilization of calcium stores, and activation of an endogenous Ca^{2+}-dependent Cl^- current. Thus, inward currents are observed in response to ACh.[4] Cyclopropane had little effect on the M_1 receptors even at 5 MAC (−5% ± 4%; fig. 5A).

Of interest, 1 MAC butane provided similar effects to those of 1 MAC cyclopropane on the most of the recep-

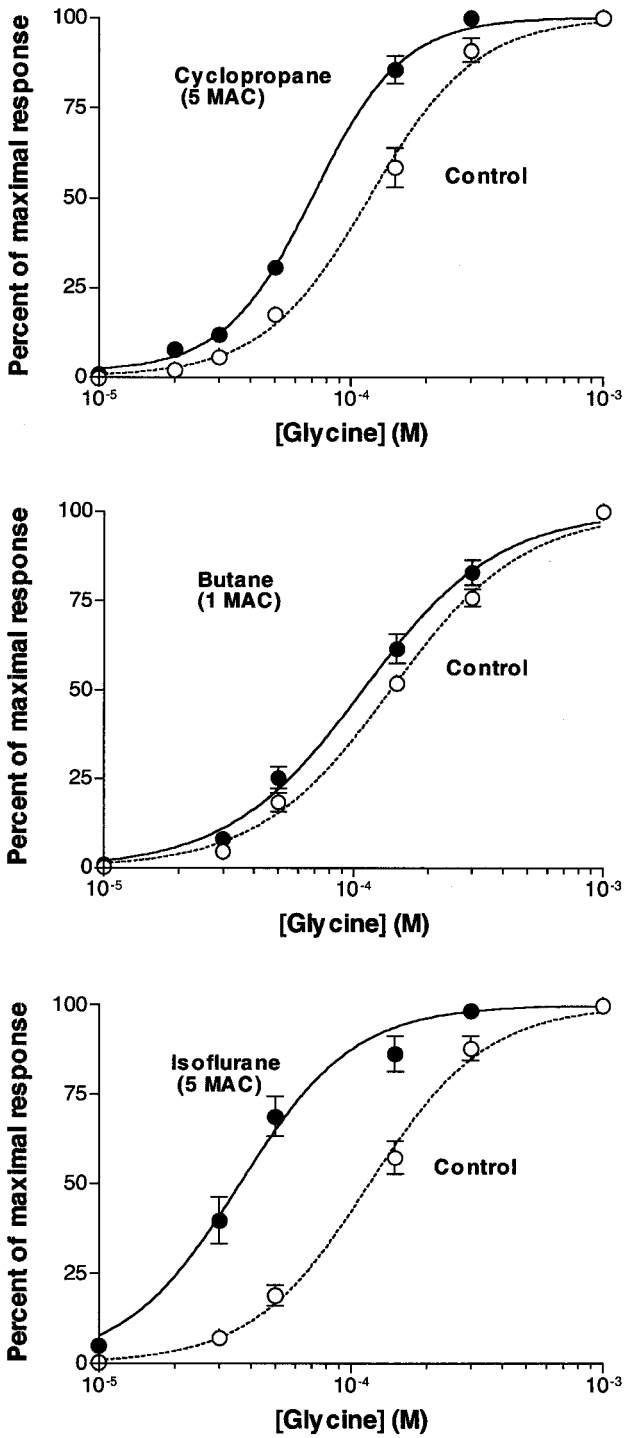

Fig. 4. Effects of anesthetics on agonist concentration–response relation for α_1 glycine receptors. Cyclopropane (5 minimum alveolar concentration [MAC]), isoflurane (5 MAC), and butane (1 MAC) significantly shifted leftward glycine concentration–response curves, indicating an increase in an apparent affinity of agonist (glycine). (*Top*) Glycine EC_{50} values were 118 μM for control and 69 μM for cyclopropane ($P < 0.05$), and Hill coefficients were 2.0 for control and 2.3 for cyclopropane (n = 6). (*Middle*) Glycine EC_{50} values were 142 μM for control and 109 μM for butane ($P < 0.05$), and Hill coefficients were 1.6 for control and 1.6 for butane (n = 8). (*Bottom*) Glycine EC_{50} values were 126 μM for control and 31 μM for isoflurane ($P < 0.05$), and Hill coefficients were 1.8 for control and 1.6 for isoflurane (n = 5). Error bars represent SEM.

tors studied (nACh receptors, −72% ± 4%; 5-HT_{3A} receptors, −3% ± 2%; NMDA receptors, −30% ± 6%; GIRK1/2 channels, 7% ± 1%; M_1 receptors, 2% ± 3%; fig. 5B). In contrast, butane had little effect on the AMPA receptors (−2% ± 3%).

Effects of Cyclopropane on $GABA_A$ Mutant Receptors

To test the idea that the small size of cyclopropane precludes its binding to serine 270 in GABA α_2 subunit, we tested five α_2 mutant $GABA_A$ receptors coexpressed with β_1 subunit with increasing volumes of amino acids at position 270. EC_5 values were determined for each of the $GABA_A$ mutant receptors, which were 0.2–0.5 μM for α_2(S270T)β_1, 0.4–0.8 μM for α_2(S270N)β_1, 0.5–0.8 μM for α_2(S270I)β_1, 0.3–0.7 μM for α_2(S270Y)β_1, and 0.02–0.075 μM for α_2(S270W)β_1 receptor. Cyclopropane (5 MAC) enhanced four of five mutants to a similar extent as $\alpha_{2\beta1}$ wild type, and did not affect α_2(S270W)β_1 receptors (fig. 6).

Neither cyclopropane (1–5 MAC) nor butane (1 MAC) produced current in any receptor studied (in the absence of neurotransmitter). Isoflurane as well as halothane (up to 5 MAC) had no effect on basal current of the glycine receptors.

Discussion

In this study, we found marked difference in the effects of nonhalogenated alkane anesthetics between $GABA_A$ and glycine receptors, *i.e.*, they enhanced glycine receptors but not $GABA_A$ receptors at clinical concentrations. The lack of the effect on $GABA_A$ receptors is consistent with a previous report.[9] It has been shown that most volatile anesthetics markedly enhance both $GABA_A$ and glycine receptors,[29–31] although some anesthetics, such as pentobarbital, propofol, and etomidate, are more effective on $GABA_A$ receptors than glycine receptors.[29,32,33] Neither $GABA_A$ receptors nor glycine receptors are affected by anesthetics such as ketamine.[34] In this context, nonhalogenated alkane anesthetics cyclopropane and butane belong to a distinct category. This result leads to the question of the mechanism underlying the differential effects of these anesthetics on $GABA_A$ and glycine receptors. Our results with glycine α_1(S267C) receptors suggest that cyclopropane interacts with the putative anesthetic and ethanol binding pocket composed of serine 267.[25–27] A small change of volume from serine (volume of the residue: 53 $Å^3$) to cysteine (65 $Å^3$) abolished the potentiation by cyclopropane. The molecular volume of cyclopropane and butane are 70 and 99 $Å^3$, respectively, and are relatively small compounds compared to other representative anesthetics, such as isoflurane (144 $Å^3$) and halothane (110 $Å^3$). We hypothesized that the volume of the putative binding

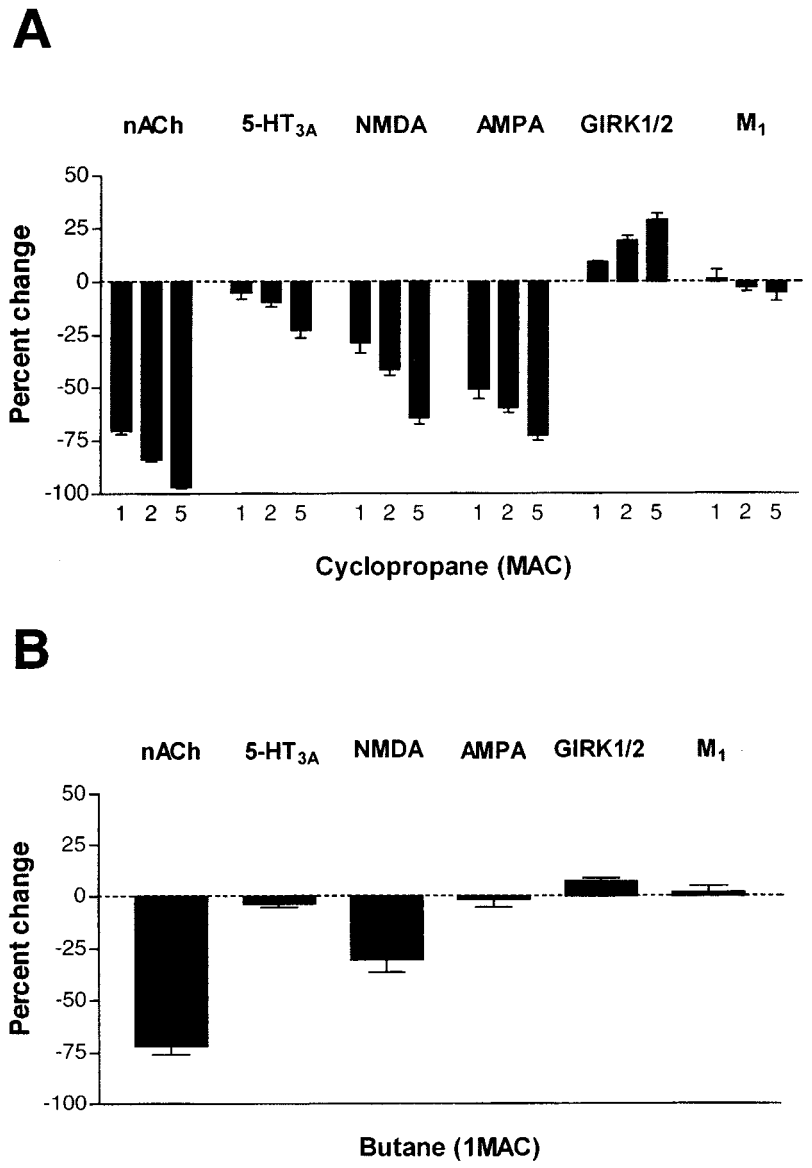

Fig. 5. Effects of nonhalogenated alkanes on recombinant receptors, including $\alpha_4\beta_2$ nicotinic acetylcholine (nACh), 5-hydroxytryptamine$_{3A}$ (5-HT$_{3A}$), NR1/NR2A *N*-methyl-D-aspartate (NMDA), GluR1/GluR2 α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), muscarinic$_1$ (M$_1$) receptors, and G-protein–coupled inwardly rectifying potassium (GIRK1/2) channels. Cyclopropane (*A*) at 1, 2, and 5 minimum alveolar concentration (MAC) and butane (*B*) at 1 MAC were tested on the oocytes expressing receptors. Equipotent concentration (1 MAC) of cyclopropane and butane provided similar effects on all receptors with an exception of the AMPA receptors. Error bars represent SEM; n = 5–9 oocytes.

pocket of $GABA_A$ receptor may be larger than that of glycine receptor and that because molecular volumes of cyclopropane and butane are small, they might not be able to interact with $GABA_A$ receptors as tightly as glycine receptors and may not exert an effect strong enough to cause conformational change on the $GABA_A$ receptors. This "critical volume" hypothesis for anesthetic actions on $GABA_A$ receptors is based on the work of Jenkins *et al.*,[35] who proposed that the volume of the binding cavity is 250–370 Å^3. Therefore, we attempted to augment the enhancing effect of cyclopropane on the $GABA_A$ receptor by a substitution of serine at position 270 in the α_2 subunit with larger amino acids, including threonine (70 Å^3), asparagine (71 Å^3), isoleucine (100 Å^3), tyrosine (116 Å^3), and tryptophan (137 Å^3). Contrary to our hypothesis, increasing the volume of the side chain did not produce significant potentiation. Conversely, the potentiation was switched to inhibition in the α_2(S270W)β_1 receptors. In a previous study,[35] a small anesthetic, chloroform (90 Å^3), provided much greater enhancement when the volume of amino acid at position 270 was increased by approximately 20–30 Å^3. Because cyclopropane is 20 Å^3 smaller than chloroform, we predicted that an increase of 40–50 Å^3 would augment the action of cyclopropane. However, we increased the volume of the side chains at position 270 by 17, 18, 47, 63, and 84 Å^3, with no evidence of enhancement of action. As a result, the small volume of nonhalogenated alkane anesthetics does not appear to be the only determinant of the insensitivity of the $GABA_A$ receptor. Because the putative binding pockets on the glycine and $GABA_A$ receptors are supposed to be lined with many different amino acids, the binding environment for cyclopropane may be different between the glycine and $GABA_A$ receptors. It also should be noted that neither cyclopropane nor butane has a dipole mo-

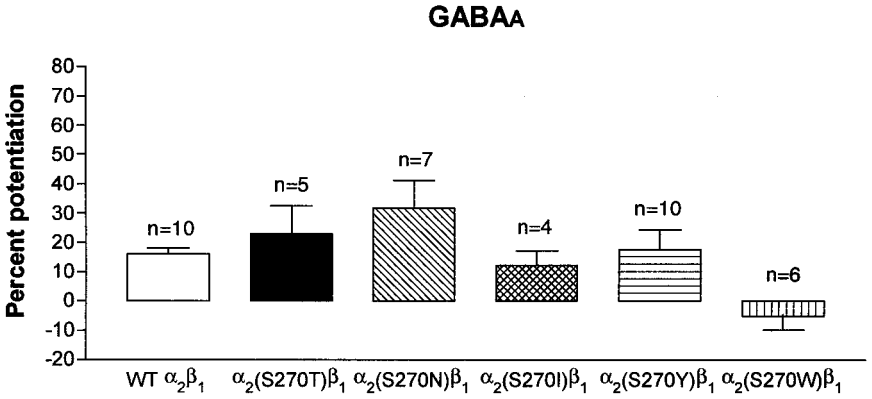

Fig. 6. Effects of cyclopropane (5 minimum alveolar concentration [MAC]) on $GABA_A$ α_2 subunit mutants receptors coexpressed with β_1 subunit. Serine at position 270 in the α_2 subunit was substituted with amino acid with increasing volume of the side chain, *i.e.*, threonine (S270T), asparagine (S270N), isoleucine (S270I), tyrosine (S270Y), and tryptophan (S270W). Except α_2(S270W)β_1 receptor, none of the mutant receptors showed any significant difference from the $\alpha_2\beta_1$ wild-type receptors. Error bars represent SEM; n = 4–10 oocytes.

ment. Perhaps polarity is more important for the binding of anesthetics to $GABA_A$ receptors than glycine receptors.

Our results showed that cyclopropane and butane affect other receptors. These anesthetics inhibited the nACh and NMDA receptors, slightly potentiated the GIRK1/2 channels, and did not change the 5-HT_{3A} and M_1 receptors. For the AMPA receptors, only cyclopropane produced a marked inhibition. $GABA_A$ receptors are thought to be a primary target of anesthetics because most volatile and nonvolatile anesthetics augment the channel activity at clinical concentrations. In terms of the nonhalogenated alkanes cyclopropane and butane, however, this is not the case. Based on sensitivity to clinical concentrations of cyclopropane and butane, glycine, nACh, and NMDA receptors are likely candidates. The nACh receptors are the most sensitive among the receptors tested in this study. Recently, nACh receptors were proposed as targets for anesthetics because volatile anesthetics and some intravenous anesthetics, such as thiopental, inhibit the function of nACh receptors.[36,37] However, nACh receptors appear not to mediate immobility *in vivo*, based on a study using a nACh receptor antagonist.[38] Furthermore, F6 (1,2-dichlorohexafluorocyclobutane), a nonimmobilizer, also inhibits nACh receptors expressed in *Xenopus* oocytes.[39] Thus, the inhibition of nACh receptors is not likely involved in immobility by anesthetics, although their role in other aspects of anesthesia remains to be elucidated.

Glycine receptors are the main inhibitory receptors in the spinal cord and brainstem, and volatile anesthetics enhance the function of these receptors. In this study, the glycine receptors were moderately potentiated by nonhalogenated alkane anesthetics. While the magnitude of the enhancing effect is less than for isoflurane or halothane, glycine receptors are probably target molecules. Indeed, this assumption is supported by *in vivo* study using rats, which demonstrated that MAC of cyclopropane is increased by an intrathecal administration of strychnine, a glycine receptor antagonist.[40]

Glutamate plays a major role in synaptic excitation in the CNS and is critical for information storage in memory and learning.[41] The NMDA receptors are known to mediate nociceptive neurotransmission in the CNS, and both the NMDA and AMPA receptors are important for memory. Inhibition of NMDA receptors is thought to be responsible for anesthetic actions of ketamine.[42] Cyclopropane and butane moderately inhibited the NMDA receptors. The AMPA receptors were more strongly suppressed by cyclopropane rather than the NMDA receptors. In contrast, butane had little or no effect on the AMPA receptors, suggesting that the suppression of the AMPA receptors is not essential for the anesthesia by nonhalogenated alkane anesthetics. Given a similarity in effects of cyclopropane and butane on most of receptors, except the AMPA receptors, a comparison of behaviors produced *in vivo* between these two anesthetics may elucidate which clinical manifestations can be explained by the AMPA receptors.

It is interesting that nonhalogenated alkane anesthetics potentiate GIRK channels, because volatile anesthetics inhibit the GIRK channels expressed in oocytes.[43] Recently, the GIRK channels were shown to be potentiated by ethanol and to be important for ethanol actions, including analgesia.[5–7] The potentiation by cyclopropane and butane may in part attribute to their analgesic actions. It is, however, unclear whether the GIRK channels are involved in immobility so far.

Previous studies showed that inhalational anesthetics have very similar effects on $GABA_A$ and glycine receptors and suggested a common site and mechanism of action for these two neurotransmitter-gated ion channels.[3,23] The current work with cyclopropane and butane provides the first evidence that $GABA_A$ and glycine receptors can be differentially sensitive to some inhalational agents. Our mutation studies suggest a common site between transmembrane domains 2 and 3 that accommodates volatile anesthetics, including cyclopropane, but this site must differ subtly between $GABA_A$ and glycine receptors.

In summary, our findings suggest that the glycine and NMDA receptors but not $GABA_A$ receptors may contribute anesthesia (immobility) produced by nonhalogenated alkanes cyclopropane and butane.

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