Isoflurane’s Enhancement of Chloride Flux through Rat Brain γ-Aminobutyric Acid Type A Receptors Is Stereoselective

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Background: Recent evidence is consistent with the view that volatile anesthetics interact directly with excitable membrane-bound channel proteins. If these agents interact directly with chiral centers in the neuronal cell membrane, then their effects should be stereoselective. Using rat brain membranes enriched in γ-aminobutyric acid type A (GABA_A) receptors, we investigated the hypothesis that the permeability response of this well-characterized central nervous system channel protein to stereoisomers of isoflurane is stereoselective.

Methods: Rat brain synaptic microvesicles were prepared by differential centrifugation. Agonist-stimulated 36Cl flux through membrane-bound GABA_A receptors was assayed in the presence of (+)- and (-)-isoflurane and compared with control conditions.

Results: Both isomers increased the potency and efficacy of GABA; however, (+)-isoflurane was significantly more potent and efficacious than the (-)-isomer. For example, the (+)-isomer (140 μM) reduced the median effective concentration of GABA from 12.7 ± 1.0 to 5.4 ± 0.5 μM, whereas the (-)-isomer reduced it to 9.6 ± 1.0 μM (P < 0.001). The (+)-isomer also was 1.6 times as potent as the (-)-isomer in augmenting 5 μM GABA-gated flux (79 ± 11 vs. 130 ± 17 μM, respectively; P = 0.01). In addition, the (+)-isomer produced significantly greater maximal enhancement of flux (9.4 ± 0.4 vs. 7.0 ± 0.3 nmol·mg⁻¹·3 s⁻¹; P < 0.001).

Conclusions: Isoflurane’s effects on GABA-gated chloride flux were stereoselective. This result supports direct interaction with a stereoselective site, possibly the GABA_A channel protein itself, rather than a nonspecific perturbation of the surrounding membrane lipid. In addition, these findings, from a functional assay using mammalian brain, agree with recent observations in invertebrate ion channels and mammalian neuronal cell cultures. (Key words: Anesthesia: mechanisms. Anesthetics, volatile: isoflurane; isoflurane stereoisomers. Central nervous system: γ-aminobutyric acid; chloride flux.)

The precise cellular site(s) and mechanism(s) of action of volatile general anesthetics have not yet been determined. Correlation of anesthetic potency with oil solubility suggests that this site is lipophilic, a property common to both membrane bilayers and membranespanning regions of channel proteins. Stereoselectivity in the actions of chiral general anesthetics may be useful to distinguish between these alternatives. For example, direct interaction with channel proteins is suggested by identification of two cation conductances in molluscan central neurons that are about twofold more sensitive to the (+)-isomer of isoflurane. The relevance of this observation was recently underscored by the finding of isoflurane’s stereoselectivity in mice tested by assays of sleep-time and minimal alveolar concentration.

In the mammalian central nervous system, a potential anesthetic target site is the γ-aminobutyric acid type A (GABA_A) receptor–chloride channel macromolecule (recently reviewed by Tanelian et al.9). This ligand-gated inhibitory receptor–channel complex contains modulatory sites for major sedatives such as barbiturates and benzodiazepines, and volatile anesthetics enhance its permeability response. The recent finding that (+)-isoflurane is more potent than the (-)-isomer in augmenting flunitrazepam binding to the GABA receptor complex from rat brain homogenates supports the hypothesis that this receptor directly interacts with isoflurane. However, these particular effects were observed only at supraphysiologic isoflurane concentrations. Furthermore, the isomers equipotently reduced binding of the GABA_A channel probe, t-butylbicyclo-
phosphorothionate, suggesting that in contrast to the benzodiazepine binding site, the GABA-activated anionic pore through which chloride ions pass failed to distinguish between the isomers. To test directly whether isoflurane stereoselectively enhances GABA channel function in a mammalian whole-brain preparation, we measured the effects of isoflurane’s purified optical isomers on GABA-gated chloride flux through rat brain synaptosomal membranes.

Materials and Methods

Approval for the study was obtained from the University of Pittsburgh’s Institutional Animal Care and Use Committee. Optical isomers of isoflurane (supplied by G. Vernicke, Anaquest, Murray Hill, NJ) were purified by capillary chromatography. The chemical purity of the isoflurane isomers was determined to be greater than 99.5% by gas chromatography, and the optical purity was greater than 99%. Male Sprague-Dawley rats (6–8 weeks old) used in this study were decapitated after brief inhalation of halothane. The entire brain was rapidly removed and kept in ice-cold buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, 10 mM glucose, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4). Fresh membrane microvesicles were prepared for each experiment by differential centrifugation. In brief, brains were gently homogenized by hand in a precooled polytetrafluoroethylene (Teflon)-coated tissue grinder (10–12 strokes), then centrifuged (900 × g for 15 min at 2°C). The resuspended pellet containing crude membranes was centrifuged at the same settings, and the resultant pellet containing microvesicles was suspended in buffer at a concentration of 6–10 mg protein/ml as assayed by the method of Hartree. Experimental results were derived from 12 separate batches of membrane microvesicles, each containing brain tissue from several animals.

Chloride influx into freshly prepared microvesicles was assayed as previously reported. Fresh membrane suspensions (200-μl aliquots) were incubated in glass test tubes partially immersed in a water bath at 34°C. A solution of chloride 36 (³⁶Cl⁻) (28.5 MBq/mmol; Amersham, Arlington Heights, IL, diluted in buffer to 74 kBq/ml), containing (+) or (-)-isoflurane (0–1.2 mM, diluted from saturated stocks of 17 mM) and GABA (0–375 μM) was added, yielding a final volume of 400 μl. At 3 s (which is within the period of linear influx), the reaction was quenched with 4 ml ice-cold buffer containing 100 μM picrotoxin (a specific chloride-channel blocker, Sigma, St. Louis, MO) and then rapidly vacuum-filtered through glass-fiber filters (Whatman GF-C, Sigma) in a filter manifold (Hoefer, San Francisco, CA). Filters were rinsed with 8 ml ice-cold buffer containing 100 μM picrotoxin to remove nonspecifically adherent ³⁶Cl⁻ and then dissolved in scintillation cocktail and counted with 100% efficiency over the energy spectrum for ³⁶Cl⁻. Flux values are expressed in nanomoles of chloride, normalized to the membrane protein content and the assay time.

Experiments were performed at a single concentration of GABA (5 μM) and six concentrations of (+)- and (-)-isoflurane from 40 to 1,700 μM to determine the potency with which the isomers of isoflurane increased GABA-gated chloride flux. Specific enhancement by isoflurane was calculated by subtracting flux values obtained in the absence of isoflurane. The effect of each of isoflurane’s isomers on GABA’s potency for stimulating chloride flux was assayed in experiments using a single concentration of isoflurane in the therapeutic range (140 μM, corresponding to a gas phase concentration of approximately 25% of the minimum alveolar concentration) and eight concentrations of GABA up to the saturating range with respect to flux (0–375 μM). GABA-gated chloride flux was calculated by subtracting values obtained in the absence of GABA and isoflurane. Controls to determine the effect of the isoflurane isomers on non-GABA-mediated chloride flux were performed with membrane preincubated with picrotoxin (100 μM) a GABA channel blocker. Concentrations of isoflurane isomers present at the moment of quenching in matched reaction mixtures lacking radiotracer were confirmed by gas chromatography (8500 gas chromatograph, Perkin-Elmer, Norwalk, CT; Poropak P column packing, Waters, Milford, MA).

Data from separate experiments were fit by an iterative nonlinear least-squares fitting routine to a logistic function of the form:

\[ y = \frac{A}{1 + e^{b(x-C)}} \]

where A = the maximal flux; b = the slope of the concentration-flux response; and C = the median effective concentration (EC₅₀), yielding estimates of the above parameters and their respective standard errors. Data were then combined and fit to similar curves. Groups corresponding to controls and each isomer were statistically compared by a method requiring the fewest assumptions about the distribution of the errors around the means derived from the normal distribution.

Results

In the absence of isoflurane, the specific chloride permeability (Pₑ) was similar in a step, sigmoid, concentration (μM) (fig. 1). The EC₅₀ was determined for each concentration of GABA by fitting a logistic function to the data. EC₅₀ (μM) and (P₀) (mmol·kg⁻¹·h⁻¹·μM⁻¹) are shown in figure 1. Based on the EC₅₀ (μM) (fig. 1C) and the specific chloride permeability (Pₑ), the concentration of isoflurane (μM) was chosen to slightly lower than the EC₅₀ for each concentration of GABA to determine the effect of the isoflurane isomers on the enhancement of GABA’s potency for stimulating chloride flux.

Both isomers of isoflurane increased GABA’s potency for stimulating chloride flux. (+)- and (-)-isoflurane produced leftward, parallel shifts in the GABA dose-response curve, decreasing the GABA concentration required to achieve 50% of the maximum response (EC₅₀) by 5.4 ± 0.5 μM (P < 0.01, fig. 1B) and 6.9 ± 1.0 μM (P = 0.05, fig. 1A), respectively (table 1). Increases in EC₅₀ represent an increase in potency by a factor of 2.3 and 3.3, respectively.

Anesthesiology. V 83, No 5, Sep 1995
STEREOSELECTIVE EFFECTS OF ISOFLURANE ON CHLORIDE FLUX

Results

In the absence of isoflurane, GABA increased channel-specific chloride permeability of rat brain microvesicles in a steep, sigmoid, concentration-dependent manner (fig. 1). The \(EC_{50}\) was 12.7 \(\pm 1.0\) \(\mu\)M, and the plateau value for GABA-gated flux was 30.4 \(\pm 0.8\) nmol \(\cdot\) mg \(^{-1}\) \(\cdot\) s \(^{-1}\). Based on these data, we chose a GABA concentration slightly less than the \(EC_{50}\) (i.e., 5 \(\mu\)M) for subsequent experiments, to determine the effect of the isoflurane isomers on GABA's efficacy.

Both isomers of isoflurane increased the apparent potency and the efficacy of GABA. Concentrations of both (+)- and (-)-isoflurane in the therapeutic-range (140 \(\mu\)M) produced leftward, parallel shifts of the [GABA]-flux curve, decreasing the GABA \(EC_{50}\) from 12.7 \(\pm 1.0\) to 5.4 \(\pm 0.5\) \(\mu\)M \((P < 0.001\) compared with GABA alone) and 9.6 \(\pm 1.0\) \(\mu\)M \((P = 0.03\) compared with GABA alone), respectively (table 1 and fig. 1). These decreases in \(EC_{50}\) represent an increase in apparent GABA potency by a factor of 2.3 and 1.3 for the (+)- and (-)-isomers, respectively. The (+)-isomer caused a significantly greater increase in GABA potency than the (-)-isomer \((P < 0.001)\). A modest increase in the maximal flux value was evident with the (-)-isomer (from 30.4 \(\pm 0.8\) to 34.9 \(\pm 1.2\), \(P = 0.002\)) but not the (+)-isomer (from 30.4 \(\pm 0.8\) to 32.5 \(\pm 0.9\), \(P = 0.11\)). Even if the [GABA]-flux curves generated in the presence of each isomer are normalized to the control maximal flux value, the apparent potency of GABA is still significantly greater in the presence of the (+)-isomer (now 4.4 \(\pm 0.6\) \(\mu\)M vs. 6.8 \(\pm 0.4\) \(\mu\)M for the (-)-isomer; \(P < 0.001\)).

Both isoflurane isomers also increased GABA efficacy; the (+)- and (-)-isomers increased 5 \(\mu\)M isoflurane-gated chloride flux in a concentration-dependent manner (fig. 2 and table 2). These effects occurred at concentrations well within the therapeutic range and represent up to 70% and 100% greater efficacy (– and +, respectively) than with GABA alone at the lower concentrations. The slopes of the [isoflurane]-flux enhancement curves were statistically indistinguishable \((P = 0.25)\). Specifically, the (+)-isomer is more than 1.6 times more potent than the (-)-isomer (79 \(\pm 11\) vs. 130 \(\pm 17\) \(\mu\)M, respectively; \(P = 0.01\)) (table 2 and fig. 2). In addition, the (+)-isomer possesses significantly greater efficacy, as indicated by a greater effect on maximal flux enhancement (9.4 \(\pm 0.4\) vs. 7.0 \(\pm 0.3\), \(P < 0.001\)).

Discussion

Our data in the absence of isoflurane are consistent with existing studies using the 3-s \(^{35}\)Cl permeability
assay, which has proven to be useful for screening for drug effects at the GABA<sub>A</sub> receptor. Our GABA EC<sub>50</sub> (12.7 ± 1.0 μM) (table 1) is comparable to previously published EC<sub>50</sub>s for GABA in similar permeability assays (range 10-13 μM). The plateau value for GABA-gated flux is somewhat greater than the maximal GABA-gated flux value previously reported in mice, but in close agreement with our recently published values. The shift of the GABA concentration-flux curve to the left in the presence of isoflurane that we observed, together with the lack of a significant effect on maximal GABA-gated chloride flux, confirms previous electrophysiological studies with millimolar concentrations of halothane and enflurane.

Anesthetic stereoisomers have been used by many groups as a tool in the search for a site(s) of volatile anesthetic action. Stereofluctivity is typically interpreted as supporting drug interaction with chiral centers in excitable membrane proteins, rather than interaction with the nonchiral interior of membrane bilayers. Among the clinical volatile agents, halothane stereoisomers were studied in vitro more than two decades ago in two model systems, but they did not differ in their ability to depress synaptic transmission or increase the molecular motions of fatty acid chains. Although the optical purity of the halothane used was not as great as that used in the current series, it seems unlikely that this would obscure some stereoselective effect. More recently, isoflurane’s isomers were also found to be equipotent in perturbing lipid bilayers in decreasing heart rate, prolonging atrioventricular conduction time, and inducing myocardial depression; in inhibiting binding of antagonists to their site on long-lasting, high-threshold calcium channels; and in inhibiting the voltage-gated fast transient potassium current as well as the sustained residual current of GABA<sub>A</sub>, channels previously exposed to desensitizing GABA concentrations.

Other electrophysiologic studies of isoflurane isomers are more consistent with recent reports of stereoelectivity in the hypnotic effect of isoflurane in mice and are thus more likely to be mechanistically important. For example, the (+)-isomer was approximately twice as efficacious as the (-)-isomer in augmenting currents in cultured rat cerebellar Purkinje cells induced by low concentrations of GABA (2 μM), at isoflurane concentrations up to approximately 1 nm. Similarly, the (+)-isomer approximately doubled the time to half decay of the GABA-mediated inhibitory postsynaptic current in cultured rat hippocampal neurons. We found that isoflurane’s isomers augment GABA-gated chloride flux with a (+);(-) potency ratio of 1.6:1 in a mammalian whole-brain preparation that is composed of all neuronal cell types and GABA<sub>A</sub> receptor subtypes that could be involved in the volatile anesthetic hypnotic response. This potency ratio corresponds exactly to that found by MAC determination in rats. Such findings buttress the growing body of data supporting that the GABA<sub>A</sub> receptor is an important site of volatile anesthetic action.

Table 2. Effect of (+) and (-) Isoflurane on 5 μM GABA-gated Flux

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<tr>
<th>Isoflurane Isomer</th>
<th>Isoflurane EC&lt;sub&gt;50&lt;/sub&gt; (μM) (±SE)</th>
<th>Maximal Flux (mmol·mg&lt;sup&gt;-1&lt;/sup&gt;·3 s&lt;sup&gt;-1&lt;/sup&gt;) (±SE)</th>
<th>Slope (±SE)</th>
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<tr>
<td>(-) Isoflurane</td>
<td>130 ± 17&lt;sup&gt;*&lt;/sup&gt;</td>
<td>7.0 ± 0.3&lt;sup&gt;†&lt;/sup&gt;</td>
<td>2.5 ± 0.7&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>(+) Isoflurane</td>
<td>79 ± 11&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>9.4 ± 0.4&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;‡&lt;/sup&gt;</td>
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Specific enhancement of 5 μM GABA-gated chloride flux by isoflurane was calculated by subtracting flux values obtained in the absence of isoflurane. These data (illustrated in fig 2) were fit to logistic functions by an iterative nonlinear least-squares fitting routine, yielding estimates of the EC<sub>50</sub>, maximal flux, slope, and their respective standard errors. Groups corresponding to each isomer were statistically compared as in table 1.

* P < 0.01, (-) versus (+).
† P < 0.001, (-) versus (+).
‡ P < 0.05, (-) versus (+).

References
STEREOSELECTIVE EFFECTS OF ISOFLURANE ON CHLORIDE FLUX

Despite these data, the precise mechanism by which isoflurane stereoselectively enhances GABA-gated chloride flux remains to be defined. Electrophysiologic data indicate that volatile anesthetics such as isoflurane can enhance chloride permeability in the presence of low GABA concentrations, but they decrease the residual steady-state chloride permeability response if GABA is present in high concentrations. In principle, differential enhancement could be due to isomer-specific effects on peak chloride ion permeability, desensitization of the GABA \(_\text{A}\) receptor complex, or both. The three- isomeric assay that we used cannot resolve these processes, which occur on the millisecond time scale. In addition, it is unknown whether chloride flux is linear with respect to time over the full range of GABA and isoflurane concentrations possible. For examples, although linearity has been confirmed for GABA alone at concentrations to 30 μM, specific information is available on the linearity of flux in the additional presence of isoflurane. However, our findings suggest that further mechanistic studies using both rapid-kinetic and electrophysiologic methods are warranted, to determine how isoflurane's stereoisomers differentially enhance GABA-gated chloride permeability.

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References