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Short communication

Stereoselective actions of halothane at $GABA_A$ receptors

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Abstract

Isoflurane anesthesia exhibits stereoselectivity, and a corresponding stereoselectivity ((+)->(−)-isomer) has been reported at $GABA_A$ receptors in vitro. The objective of the present study was to determine if the positive modulatory actions of halothane at $GABA_A$ receptors exhibited a similar stereoselectivity. Both (*R*)- and (*S*)-halothane ((+)- and (−)- isomers, respectively) enhanced [^{3}H]flunitrazepam binding to brain membranes in a concentration dependent manner without a significant difference in either potency (EC_{50}) or efficacy (E_{max}). While both (*R*)- and (*S*)-halothane enhanced [^{3}H]muscimol binding, the potency of the (+)-isomer was slightly greater than the corresponding (−)-isomer (0.91 ± 0.17 versus 1.45 ± 0.04% atmospheres, respectively ($P < 0.02$)). Thus, subtle structural differences between inhalational anesthetics can have a significant impact on the degree of stereoselectivity at the receptor level and may provide insights for the development of more specific drugs. © 1998 Published by Elsevier Science B.V.

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1. Introduction

The molecular mechanisms responsible for the anesthesia produced by inhalational agents remains controversial, but converging lines of evidence suggest that proteins, and in particular, ion channels may be the primary targets of these agents (Franks and Lieb, 1994; Moody et al., 1994b). Pharmacologically relevant concentrations of inhalational anesthetics can modulate both ligand- and voltage-operated ion channels including $GABA_A$ receptors, K^+ channels, NMDA (*N*-methyl-D-aspartate) receptors, and L-type Ca^{2+} channels (Moody et al., 1988, 1994a; Franks and Lieb, 1991; Martin et al., 1991). The demonstration that the optical isomers of isoflurane exhibit modest, albeit statistically significant differences in anesthetic potency in vivo (Harris et al., 1992; Lysko et al., 1994) is fully consistent with the hypothesis that isoflurane interacts at specific protein targets in the central nervous system (Franks and Lieb, 1994; Moody et al., 1994b). Given these data, the production of safer anesthetics using optically active agents is not only a possibility (Moody et al., 1994b), but also provides tools for discriminating among relevant loci of anesthesia. Thus, if stereoselectivity is not manifested at a putative target of anesthesia in vitro, then it is less likely to be involved in the anesthetic process than a locus exhibiting stereoselectivity (Moody et al., 1994a).

Both electrophysiological and radioligand binding studies have demonstrated a stereoselective action of isoflurane isomers at $GABA_A$ receptors ([+] > [−]isoflurane) (Jones and Harrison, 1993; Moody et al., 1993, 1994b; Harris et al., 1994). Several other clinically useful inhalational agents possess a center of asymmetry. The purpose of this study was to determine if the stereoselectivity at $GABA_A$ receptors manifested by isoflurane was common to the structurally related inhalational agent, halothane. Due to very limited quantities of the halothane isomers available, these studies were performed using radioligand binding assays previously demonstrated to be sufficiently sensitive to detect stereoselectivity between (+)- and (−)-isoflurane (Moody et al., 1993; Harris et al., 1994).

2. Materials and methods

2.1. Tissue preparation and radioligand binding assays

These methods are identical to those previously published (Moody et al., 1993; Harris et al., 1994). In brief,

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male (25–30 g) NIH mice (Veterinary Resources Branch, Bethesda, MD) were group housed in a vivarium for at least one week prior to use. Animals were maintained in a 12 h light cycle (lights on 07:00) with water and rat chow freely available. Cerebral cortices were prepared for radioligand binding studies as previously described (Moody et al., 1993). Animals were killed by decapitation, and fresh cerebral cortices were removed and disrupted in 50 volumes of ice-cold 50 mM Tris–citrate buffer (pH 7.4) using a Brinkmann Polytron. The tissues were then centrifuged at $20{,}000 \times g$ for 20 min and resuspended in 50 volumes of buffer.

2.2. Radioligand binding assays

[^{3}H]Flunitrazepam binding: tissues were 'washed' (resuspended/recentrifuged as described above) a total of five times with the final tissue pellet resuspended in 8–10 volumes of buffer. Assays were performed using sealed 1 ml microtiter plates (Beckman Instruments, Columbia, MD) in a total volume of 750 μl containing: 100 μl tissue suspension ($\sim$0.4 mg protein/assay), 50 μl radioligand (Sp. Act. 83.4 Ci/mmol; NEN–Dupont, Boston, MA), and drug solution or buffer to volume. Nonspecific binding was defined using flumazenil (10 μM) and constituted $\sim$2–5% of total binding. Anesthetics were added as a saturated solution in 50 mmol Tris–citrate buffer, and their concentrations determined by gas chromatography.

A similar protocol was used to determine [^{3}H]muscimol (Sp. Act. 20.0 Ci/mmol; NEN–Dupont, Boston, MA) binding. Nonspecific binding was defined with GABA (1 mM) and was $\sim$20% of total binding. Assays were performed in sealed 1 ml microtiter plates (Beckman Instruments, Columbia, MD). Assays were performed in duplicate or triplicate, and all experiments repeated 5–6 times. Reactions were terminated after 30 min (4°C) by rapid filtration over GF/B filters using a Brandel M-48R (Brandel Instruments, Gaithersberg MD) followed by washing with two 5 ml aliquots of ice-cold Tris–citrate buffer. The radioactivity retained on the filters was measured with a Beckman LS 5801 liquid scintillation counter. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL).

2.3. Determination of anesthetic concentrations

Gas chromatography was used as previously described (Moody et al., 1994a). Using a gas-tight Hamilton syringe, the assay buffer was sampled directly through a nonpermeable membrane and injected into the gas chromatograph (HP 5880-A) with an HP-1 column. All measurements were carried out under assay conditions. Anesthetic concentrations were calculated by linear regression utilizing the peak areas obtained from known standard concentrations with the same injection volume (2 μl) to those obtained from each sample.

2.4. Data analysis

Data were analyzed by nonlinear regression using Inplot4 (Graphpad Software, La Jolla, CA). Statistical comparisons of EC_{50}, E_{max} were performed using Student's *t*-test (Instat, Graphpad Software, La Jolla, CA).

2.5. Materials

Radioligands were obtained from NEN–Dupont (Boston, MA). The stereoisomers of halothane were kindly supplied by Dr. Meinwald (Ithaca, NY) and prepared according to published methods (Meinwald et al., 1991). GABA and muscimol were purchased from Sigma (St. Louis, MO). Flumazenil was donated by Hoffmann–LaRoche (Nutley, NJ). Other materials were obtained from standard commercial sources.

3. Results

As anticipated from previous studies with racemic halothane and the enantiomers of isoflurane (Moody et al., 1993; Harris et al., 1994), the stereoisomers of halothane increased [^{3}H]flunitrazepam in a concentration dependent fashion. However, no stereoselectivity was manifested in this measure. The EC_{50} values for the enhancement of [^{3}H]flunitrazepam were 0.35 ± 0.05 and 0.31 ± 0.14% atmospheres for the (*R*)- and (*S*)-enantiomers, respectively. The corresponding E_{max} (maximum enhancement) values were 67 ± 6 and 71 ± 4%, respectively (Table 1; Fig. 1a).

Both halothane enantiomers also enhanced [^{3}H]muscimol binding in a concentration dependent manner. (*R*)-Halothane was $\sim$60% more potent the (*S*)-isomer. The EC_{50} values were 0.91 ± 0.17 and 1.45 ± 0.04% atmo-

Table 1
Halothane stereoisomers enhance [^{3}H]flunitrazepam and [^{3}H]muscimol binding

	EC_{50} (% atmosphere)	E_{max} (% enhancement)
[^{3}H]flunitrazepam binding		
(*R*)-(+)-Halothane	0.32 ± 0.05	67 ± 6
(*S*)-(−)-Halothane	0.31 ± 0.14	71 ± 4
[^{3}H]muscimol binding		
(*R*)-(+)-Halothane	0.91 ± 0.17*	40 ± 5
(*S*)-(−)-Halothane	1.45 ± 0.04	34 ± 2

Studies were performed as detailed in Section 2 ($n = 5$). Halothane concentrations varied from 0 to 2%. The EC_{50} values are significantly different ($P < 0.02$) but the E_{max} values are not for [^{3}H]muscimol binding.

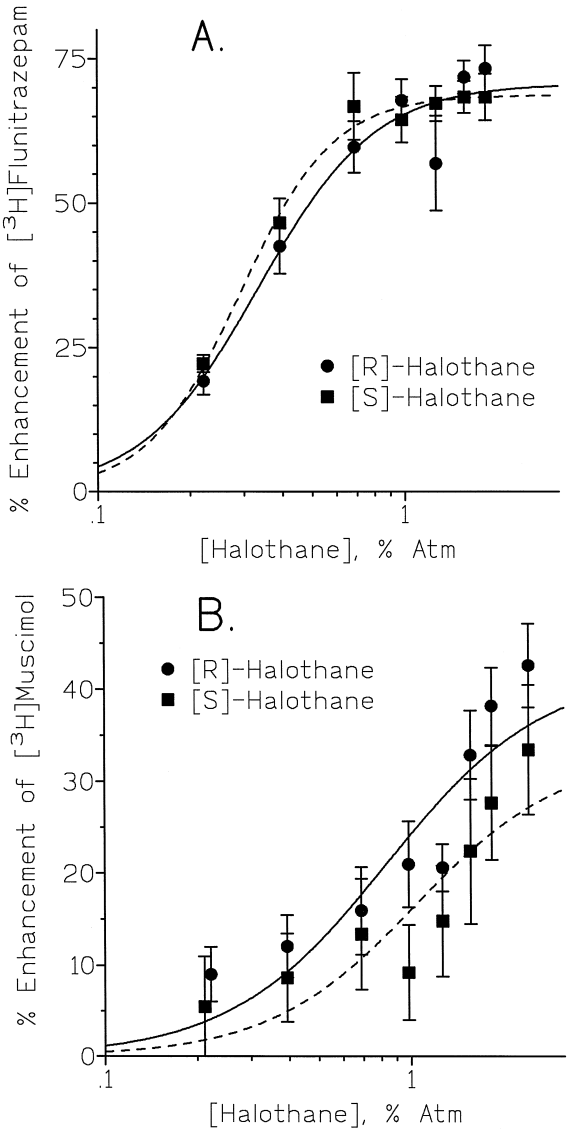

Fig. 1. (A) Halothane lacks stereospecificity in the enhancement of [^{3}H]flunitrazepam binding. Experiments were performed as detailed in Section 2. The EC_{50} and E_{max} values were 0.32 ± 0.05 and 0.31 ± 0.14% atmospheres ($P > 0.05$) and 67 ± 6 and 71 ± 4% ($P > 0.05$) enhancement of basal binding for (*R*)- and (*S*)-halothane respectively. $n = 5$. (B) Halothane demonstrates stereoselectivity in the enhancement of [^{3}H]muscimol binding. Experiments were performed as detailed in Section 2. The EC_{50} and E_{max} values were 0.91 ± 0.17 and 1.45 ± 0.04 ($P < 0.02$) and 40 ± 5 and 34 ± 2% ($P > 0.05$) enhancement for (*R*)- and (*S*)-halothane respectively. $n = 5$.

spheres for (*R*)- and (*S*)-halothane, respectively ($P < 0.02$). No significant difference was observed in the corresponding E_{max} values (40 ± 5 and 34 ± 2% increases, respectively) (Table 1; Fig. 1b).

4. Discussion

Both stereoisomers of halothane produced concentration dependent increases in [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Table 1). These findings are consistent with the previously reported effects of racemic halothane (Nakao et al., 1991; Harris et al., 1994). While there were no differences in either the potencies or efficacies of these enantiomers to enhance [^{3}H]flunitrazepam binding, a modest (~60%), albeit statistically significant difference in potency was observed with respect to enhancement of [^{3}H]muscimol binding ((*R*)->(*S*)-halothane) (Table 1). Although the analysis may be limited both by the low potency of these anesthetics and the concentrations of halothane used (2% atmospheres), no difference in the maximum increase, i.e. efficacy, in [^{3}H]muscimol binding was observed between the stereoisomers. This finding is consistent with previous studies demonstrating equal efficacy in the enhancement of [^{3}H]muscimol binding with the isomers of isoflurane (Harris et al., 1994). Furthermore, these assays were performed in the same manner in which we previously demonstrated stereoselectivity with isoflurane (Moody et al., 1993; Harris et al., 1994). The (*R*)-isoflurane enantiomer was ~2-fold more potent than the (*S*)-isoflurane enantiomer in modulating both [^{3}H]flunitrazepam and [^{3}H]muscimol binding (Moody et al., 1993; Harris et al., 1994). Thus, stereospecific modulation of $GABA_A$ receptors by isoflurane is more robust than halothane.

In several other model systems, stereoselectivity with halothane was not observed. However, these studies were done with incompletely resolved isomers. These halothane stereoisomers equally affected the electron spin resonance of phospholipid bilayers and the helical conformation of hemoglobin (Laasberg and Hedley-White, 1971; Kendig et al., 1973). Depression of synaptic transmission in cervical ganglion by halothane was also equipotent with halothane enantiomers (Laasberg and Hedley-White, 1971). This lack of stereospecificity may be attributable to both the impure isomer separation employed, and the intrinsically limited stereoselectivity exhibited by halothane. However, utilizing purified enantiomers, other investigators have previously reported both halothane stereoisomers and halothane racemate produce differential effects on mobility (the endpoint used as a surrogate for anesthesia) in the nematode *Caenorhabditis elegans* in both wild type and various genetic mutants (Sedensky et al., 1994). The relationship between these observations and the current study is unknown.

The stereoisomers of isoflurane exhibit a significant difference in anesthetic potency (~60% difference in anesthetic MAC (minimum alveolar concentration) in rats) (Lysko et al., 1994), but it remains unknown whether the isomers of halothane exhibit a corresponding potency difference. Nonetheless, if activation of $GABA_A$ receptors contributes to the anesthesia produced by these inhalational agents, then based on the relatively modest stereoselectivity of halothane in these in vitro measures, it can be predicted there would be a correspondingly smaller difference manifested in vivo. Whether the current methods

(Lysko et al., 1994) used are sufficiently sensitive to detect such a difference in mammals with halothane awaits experimental investigation.

These data demonstrate the effects of the stereoisomers of halothane in a mammalian system. In concert with previously published data (Moody et al., 1993; Harris et al., 1994), the structure of inhalational anesthetics determines the degree of stereoselectivity exhibited by a given agent. Thus, the structurally simple halothane molecule ($CF_3CHBrCl$) would be expected to exhibit less stereoselectivity than the more complex isoflurane molecule ($CF_3CHClOCHF_2$). This information indicates that traditional drug design and development techniques could be applied to increase both potency and selectivity of these anesthetics that possess low therapeutic indices (Wolfson et al., 1978). Nonetheless, significant limitations remain in the toxicity of volatile agents. These agents are present in all tissues of the body in high concentrations and thus have significant side-effects, such as myocardial depression. Even if anesthetics are developed possessing greater stereoselectivity ($\sim$10–100 fold) the overall effect on the therapeutic index of a single stereoisomer will not necessarily increase proportionally. The overall toxicity is likely determined by interaction at other sites where stereoselectivity may not be manifest (Graf et al., 1994; Moody et al., 1994a). Thus, the clinical utility of a stereoselective agent may be limited by both the cost of preparation and side effect profile.

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