Anesthesiology
81:124–128, 1994

Inhibition of [^{3}H]Isradipine Binding to L-Type Calcium Channels by the Optical Isomers of Isoflurane

Lack of Stereospecificity

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Background: **The dose-dependent myocardial depression of volatile general anesthetics such as isoflurane has been linked to blockade of L-type Ca^{2+} channels. The effects of (+)- and (−)-isoflurane on the inhibition of [^{3}H]isradipine binding to L-type Ca^{2+} channels in membranes prepared from mouse heart were examined. In addition, because there is a stereospecific effect of these isomers on sleep time in mice, the potential contribution of L-type Ca^{2+} channels to isoflurane-induced sleep was assessed by determining whether a similar stereoselectivity would be manifested at these sites in cerebral cortical membranes.**

Methods: **The effects of isoflurane stereoisomers on the binding of an L-type Ca^{2+} channel ligand ([^{3}H]isradipine) were studied in cardiac and brain cortical membranes. Their potencies and effects on the K_d and B_{max} of [^{3}H]isradipine were measured.**

Results: **Pharmacologically relevant concentrations of (+)- and (−)-isoflurane inhibited [^{3}H]isradipine binding. The IC_{50} values for (+)-isoflurane were 0.48 ± 0.02% and 0.40 ± 0.01% in heart and brain membranes, respectively. The values for (−)-isoflurane were not significantly different from the respective values for the (+)-isomer. Saturation analysis demonstrated (+)- and (−)-isoflurane inhibited [^{3}H]isradipine binding by significantly reducing B_{max} and increasing K_d, but there were no significant differences between these isomers in either tissue.**

Conclusions: **The stereoisomers of isoflurane are equipotent as inhibitors of [^{3}H]isradipine binding to L-type Ca^{2+} channels. This lack of stereoselectivity between (+)- and (−)-isoflurane indicates that the [^{3}H]isradipine site on L-type Ca^{2+} channels in brain does not contribute to the differences in isoflurane-induced sleep time reported for these stereoisomers. Taken with a lack of stereoselectivity at L-type Ca^{2+} channels in heart, an optically resolved isomer of isoflurane may have clinical advantages compared to the current racemic mixture. (Key words: Anesthetics, volatile: Ca^{2+} channels; isoflurane; isradipine; mechanism of action; stereoisomers.)**

This article is accompanied by an editorial. Please see: Lynch C III, Pancrazio JJ: Snails, spiders, and stereospecificity—is there a role for calcium channels in anesthetic mechanisms? ANESTHESIOLOGY 81:1–5, 1994.

SEVERAL widely used volatile anesthetics (*e.g.*, isoflurane, halothane) contain a center of asymmetry. Thus, these compounds can exist in two stereoisomeric forms that are mirror images, identical in all physical and chemical respects other than their abilities to rotate plane polarized light. Because of these properties, stereoisomeric pairs have been powerful tools in determining whether drug effects are due to receptor-mediated events.[1] Thus, the demonstration of a significant difference in potency between stereoisomers strongly suggests that such differences are due to specific receptor interactions where steric considerations are often crucial. Although chirality *per se* is not required for anesthetic action (*e.g.*, sevoflurane and diethyl ether), the stereoisomers of several volatile anesthetic have been separated (*e.g.*, halothane, isoflurane).[2,3] As

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Received from the Department of Anesthesiology and Critical Care Medicine, Cardiac Anesthesia Division, The Johns Hopkins Medical Institutions, Baltimore, Maryland, and the Laboratory of Neuroscience, National Institutes of Health, Bethesda, Maryland. Accepted for publication March 14, 1994.

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with the stereoisomers of other drug,[4,5] these optical isomers have been used as tools *in vitro* to identify potential molecular targets of anesthetic action.[6–10] Moreover, (+)-isoflurane was reported to induce significantly longer sleep times in mice than identical doses of (−)-isoflurane after a single intraperitoneal injection of drug.[11] Although the latter observation cannot be causally related to anesthesia, these preliminary findings indicate that the hypnotic potential of inhalational anesthetics such as isoflurane is stereoselective.

During the past decade, converging lines of evidence indicate that many of the pharmacologic effects of inhalational anesthetics may result from their actions on ion channels.[12] For example, pharmacologically relevant concentrations of inhalational agents such as halothane and isoflurane increase ion movement through $GABA_A$ receptor-gated chloride channels.[9,13] Moreover, the ability of isoflurane to affect $GABA_A$ receptors exhibits a modest (about twofold) stereoselectivity.[8–10] Several lines of evidence also indicate that the cardiac depression produced by volatile anesthetics is linked in part to their effects on L-type Ca^{2+} channels.[14,15] The current studies were designed to determine whether the ability of isoflurane to inhibit radioligand binding to these L-type Ca^{2+} channels in heart and brain exhibited stereoselectivity.[14,15]

Methods

Experiments were performed using adult, male National Institutes of Health (NIH) Swiss mice under American Association of Accreditation of Laboratory Animal Care/NIH-approved conditions. These studies were approved by the institutional animal use committee. Experimental protocols used for radioligand binding to L-type Ca^{2+} channels were essentially as described elsewhere.[16,17] In brief, mice were killed by decapitation, and heart and brain cortical tissues were rapidly removed and placed in 50 mM ice-cold Tris-citrate buffer (*p*H 7.4). Tissues were disrupted in 50 volumes of buffer with a Brinkman Polytron (Westbury, NY). Cortical homogenates were centrifuged once at 24,000 × g for 15 min. The resulting pellet was resuspended in 25 volumes of buffer to yield a final concentration of 0.2 mg protein/assay. Cardiac ventricular homogenates were centrifuged at 1,000 × g for 10 min and the supernatant then centrifuged at 24,000 × g for 15 min. These tissue pellets were resuspended 25 volumes to yield a final concentration of 0.1 mg protein/assay.

[^{3}H]Isradipine (Sp. Act. 70.9 Ci/mmol, Dupont-NEN, Boston, MA) binding was performed in capped, airtight 1-ml microtiter plates (Beckman, Columbia MD). The total incubation volume was 0.75 ml, consisting of 0.1 ml tissue suspension (0.1–0.2 mg protein), 0.05 ml radioligand, and anesthetic in buffer and/or buffer to final volume. Nonspecific binding determined in the presence of 10 μM nitrendipine was approximately 14% in the presence of 100 pM ligand and ranged from 6% to 30% in the saturation studies. The concentration of [^{3}H]isradipine used in competition experiments was 100 pM and 25–1,000 pM for saturation studies, respectively. Tissues were incubated at 25°C for 1 h and the reactions terminated by rapid filtration under vacuum through GF/B filters using a Brandel 48 well cell harvester (Brandel Instruments, Gaithersburg, MD). Tissues were washed twice with 5 ml of ice-cold buffer solution. The radioactivity retained on the filters was determined by liquid scintillation counting. Assays were performed in duplicate, and each experiment was repeated with six tissue preparations. Specific binding was calculated by subtracting the nonspecific binding from the total binding. Protein concentrations were determined using the bicinchoninic assay (Pierce, Rockford, IL). Isoflurane was added as an ice-cold buffer solution (20.2 mM), and drug concentrations in assays were confirmed using head space gas chromatography.[8,10] A Hewlett Packard 5880 gas chromatograph (Boulder, CO) with a 25-meter methylsilicone gum column and a 40°C oven were used. The injection temperature was 80°C, and the detector temperature was 175°C. Samples of 10 μl were injected with a gas-tight syringe. Run times were approximately 1.5 min. The peak area of the sample was used to calculate the anesthetic concentration by comparison with samples of known concentration.

Nonlinear curve fitting (Inplot4, Intuitive software, La Jolla, CA) was used to determine the IC_{50}, K_d, and B_{max} values. The IC_{50} represents the concentration of anesthetic required to inhibit specific binding by 50%. IC_{50} values were estimated using nonlinear iterative curve fitting to a sigmoid function: $Y = A + (B - A)/1 + (10^X)^D/(10^C)^D$ where A is the bottom plateau, B is the top plateau, C is the log IC_{50}, and D is the Hill coefficient. K_d represents the dissociation constant of the ligand for its binding site, and the B_{max} is the maximum binding capacity. These values were calculated by nonlinear curve fitting to a rectangular hyperbola

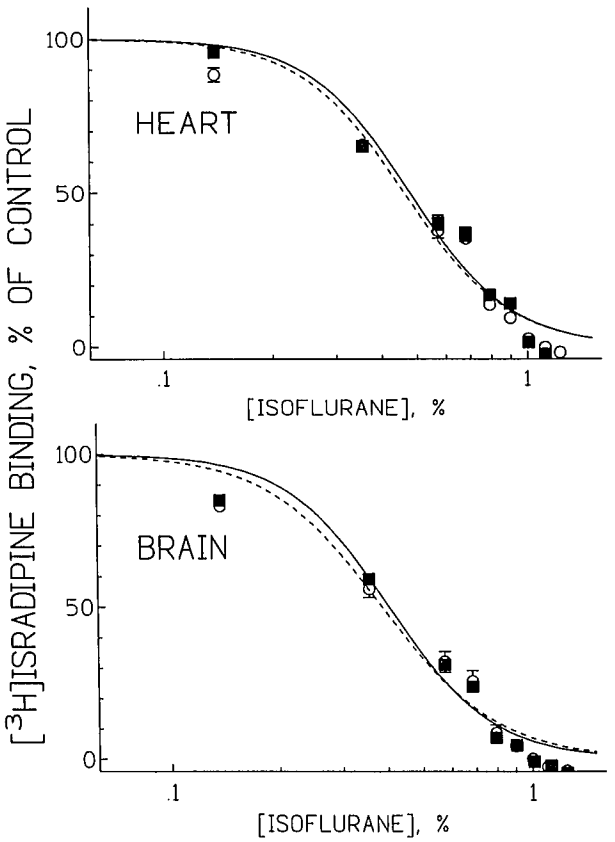

Fig. 1. Inhibition of [^{3}H]isradipine binding by (+)- and (−)-isoflurane. (*Top*) Heart. (*Bottom*) Brain. Squares and solid lines indicate (+)-isoflurane. Circles and dashed lines indicate (−)-isoflurane. Values represent the mean ± SEM for six experiments. Error bars are smaller than points in some cases. The IC_{50} values were 0.48 ± 0.02% and 0.46 ± 0.01% for (+)- and (−)-isoflurane in cardiac tissue and 0.40 ± 0.01% and 0.38 ± 0.02% in brain membranes, respectively. There were no significant differences in potency between isomers in either tissue.

using the equation $B = (B_{max})\ (L)/(K_d + L)$, where B is the amount of radioligand bound at concentration L.

Data were compared for statistical significance using an analysis of variance (Statview, Calabasas, CA).

Results

Both stereoisomers of isoflurane produced a complete, concentration-dependent inhibition of [^{3}H]-isradipine binding to brain cortical and cardiac membranes (fig. 1). The IC_{50} values for (+)- and (−)-isoflurane in brain were 0.40 ± 0.01 and 0.38 ± 0.02%, respectively. In cardiac membranes, IC_{50} values were 0.48 ± 0.02 and 0.46 ± 0.01%, respectively (fig. 1). There were no statistically significant differences in potency between these isomers in either tissue. Because these initial studies were conducted with subsaturating concentrations of [^{3}H]isradipine, additional analyses were performed with each stereoisomer (0.6%) in brain and cardiac membranes over a range of radioligand concentrations. Although both isomers significantly decreased the B_{max} and increased the K_d of [^{3}H]isradipine, there were no significant differences between the isomers in either tissue (fig. 2 and table 1).

Discussion

These studies were prompted by the recent demonstration that the optical isomers of isoflurane differentially increase sleep time in mice[11] and exhibit a modest but significant stereoselectivity at $GABA_A$ receptors in mammalian central nervous system.[8–10] Because commercially available isoflurane is a racemic (±) mixture, we examined the effects of the optically resolved isomers on [^{3}H]isradipine binding to L-type Ca^{2+} channels in cardiac membranes. This measure was chosen because the dose-dependent myocardial depression of volatile anesthetics has been linked to their effects on L-type voltage-dependent Ca^{2+} channels.[14,15] Thus, pharmacologically relevant concentrations of inhalation agents have been shown to inhibit the binding of selective, high-affinity L-type Ca^{2+} channel antagonists such as [^{3}H]isradipine to cardiac membranes, and it has been proposed that this may serve as

Table 1. Inhibition of [^{3}H]Isradipine Binding by the Optical Isomers of Isoflurane: Lack of Stereoselectivity

	K_d	B_{max}
Heart		
Control	120 ± 10	450 ± 10
(+)-Isoflurane	430 ± 60	330 ± 10
(−)-Isoflurane	400 ± 50	330 ± 30
Brain		
Control	130 ± 10	360 ± 30
(+)-Isoflurane	570 ± 80	200 ± 20
(−)-Isoflurane	530 ± 70	180 ± 20

Values represent the mean ± SEM of six tissue preparations.

Saturation analyses were performed as described in methods. K_d and B_{max} values are expressed as pmol and fmol/mg protein, respectively. At a concentration of 0.6%, both isomers significantly increased the K_D ($P < 0.01$) and decreased the B_{max} ($P < 0.05$) of [^{3}H]isradipine in heart and brain. No significant differences between isomers were manifested in either tissue.

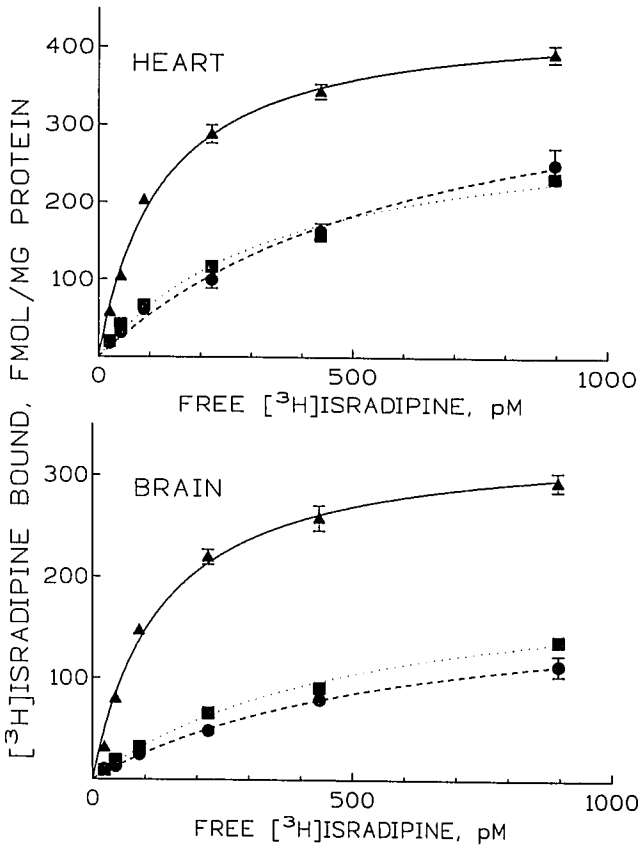

Fig. 2. Inhibition of [^{3}H]isradipine by the optical isomers of isoflurane: saturation analyses. (*Top*) Heart. (*Bottom*) Brain. Squares and solid lines indicate (+)-isoflurane. Circles and dashed lines indicate (−)-isoflurane. Values represent the mean ± SEM for six experiments. Error bars are smaller than points in some cases. Both isomers of isoflurane (0.6%) significantly increase the K_D and decrease the B_{max} of [^{3}H]isradipine. See table 1 for details.

a marker of cardiac depression.[14,15] Whereas there are significant differences in the cellular effects of anesthetics on the multiple types of Ca^{2+} channels present in cardiac tissue, the predominant mechanism of negative inotropism is thought to be alteration of Ca^{2+} influx through the L-type Ca^{2+} channel.[14] The modulation of L-type Ca^{2+} channels is thought to be the primary mechanism of myocardial depression observed with isoflurane because this volatile anesthetic has little effect on Ca^{2+} release in the sarcoplasmic reticulum.[18] Because ion channels have been postulated to be important targets of anesthetic action,[12] we also determined whether a corresponding stereoselectivity between the isomers of isoflurane would obtain for inhibition of ligand binding to L-type Ca^{2+} channels in brain cortical membranes.

The ability of (+)- and (−)-isoflurane to inhibit [^{3}H]isradipine binding to cardiac membranes (fig. 1 and table 1) is consistent with previous reports demonstrating that a variety of volatile anesthetics can affect radioligand binding to L-type Ca^{2+} channels.[14–16] Despite the lack of stereoselectivity between isomers, the potency of isoflurane reported here appears to be higher than previously reported by Drenger *et al.* for [^{3}H]nitrendipine binding to bovine heart membranes[15] and [^{3}H]isradipine binding to rat cortical membranes.[19] Because isoflurane inhibits radioligand binding to L-type Ca^{2+} channels in heart tissue both by increasing K_d (reducing the apparent affinity) and reducing B_{max} (fig. 2 and table 1), the IC_{50} value will be markedly dependent upon the radioligand concentration employed (and K_d of the particular radioligand) as well as a variety of other factors such as membrane preparation and incubation conditions.

In contrast to the current findings demonstrating that the isomers of isoflurane do not exhibit stereoselectivity at L-type calcium channels in heart or brain, we and others have demonstrated stereoselective effects of these isomers at the $GABA_A$ receptor complex using both radioligand binding and electrophysiologic techniques.[8–10] Although these potency differences are modest (~2 fold), the magnitude and order ((+) > (−)) of stereoselectivity obtained is consistent with both the differences in isoflurane-induced sleep time between these isomers reported in mice[11] and the relatively low potencies of volatile anesthetics. If isoflurane-induced inhibition of radioligand binding to L-type Ca^{2+} channels is related to depression of cardiac contractility[14] and the stereoselective difference in isoflurane-induced sleep time[11] also pertains for its anesthetic actions, then we hypothesize that a single stereoisomer of isoflurane may have clinical advantages over the currently used racemic mixture.

Whether stereoselectivity is manifested for other pharmacologic actions associated with volatile anesthetics (for example, the triggering of malignant hyperthermia or induction of halothane hepatitis) warrants further investigation. Such *in vivo* investigations with additional *in vitro* studies may be useful in determining pharmacologically relevant loci of anesthetic action as well as designing more useful inhalational agents.

The stereoisomers of isoflurane were kindly supplied by Dr. C. G. Huang, of Anaquest (Murray Hill, NJ).

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