Can the Stereoselective Effects of the Anesthetic Isoflurane Be Accounted for by Lipid Solubility?

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ABSTRACT Isoflurane is an inhalational general anesthetic widely used in surgical operations as a racemic mixture of its two optical isomers. The recent availability of pure enantiomers of isoflurane has encouraged their use in experimental studies, and stereoselective effects have now been observed on anesthetic-sensitive neuronal ion channels. Although it has been assumed that such chiral effects demonstrate direct interactions with proteins, it is possible that they could be due to stereoselective interactions with chiral membrane lipids. We have determined the partition coefficients of the two optical isomers of isoflurane between lipid bilayers and water, using racemic isoflurane and gas chromatography with a chiral column. For lipid bilayers of phosphatidylcholine (PC) and 4 mol% phosphatidic acid (PA), both with and without cholesterol (CHOL), we found equal partitioning of the isoflurane optical isomers. The ratios of the S(+) to R(−) isoflurane partition coefficients were (mean ± SEM): 1.018 ± 0.010 for bilayers of PC/CHOL/PA (mole ratios 56:40:4) and 1.011 ± 0.002 for bilayers of PC/PA (mole ratio 96:4). Molar partition coefficients for racemic isoflurane were 49 ± 4 and 165 ± 10, respectively. These findings support the view that the stereoselective effects on ion channels observed with isoflurane are due to direct actions on proteins rather than lipids.

INTRODUCTION

Volatile general anesthetics are relatively simple organic compounds which have traditionally been thought to act by "nonspecific" mechanisms (Dluzewski et al., 1983; Miller, 1985). This apparent simplicity has tended to obscure the fact that most clinically useful general anesthetics contain a chiral carbon atom. In fact, the commonly used volatile agents isoflurane, halothane, and enflurane each exist in two mirror-image (enantiomeric) forms, although these agents are always administered clinically as racemic mixtures of the isomers. It is only recently, with the preparation of optically pure enantiomers of isoflurane (Huang et al., 1992), that definitive studies using the individual isomers have become possible. On the basis of vibrational circular dichroism studies (Polavarapu et al., 1992), the (+) and (−) optical isomers of isoflurane have been assigned the absolute configurations S and R, respectively. In molluscan neurons, the S(+) isomer of isoflurane is about twice as effective as the R(−) isomer both in eliciting an anesthetic-activated potassium current and in inhibiting a neuronal nicotinic acetylcholine receptor (Franks and Lieb, 1991). S(+) isoflurane has also been found to be twice as effective as the R(−) isomer in prolonging evoked inhibitory postsynaptic currents mediated by $GABA_A$ receptor channels in cultured rat hippocampal neurons (Jones and Harrison, 1993). In addition, S(+) isoflurane enhances maximum binding of flunitrazepam to $GABA_A$ receptors in mouse cerebral cortical tissue by up to two times more than does the R(−) isomer (Moody et al., 1993). Where dose-response data are available, the stereoselective effects of isoflurane appear to be due to either differential binding (Franks and Lieb, 1991) or differential efficacy (Franks and Lieb, 1991; Moody et al., 1993) of the two enantiomers. The situation with regard to in vivo effects of isoflurane on general anesthesia is less clear. In mice, sleep time after intraperitoneal injection of low doses of isoflurane is about 50% greater for the S(+) than for the R(−) isomer (Harris et al., 1992). In tadpoles, however, it has been reported in a preliminary study that EC_{50} concentrations for loss of righting reflex are identical (Firestone et al., 1992).

The observations of stereoselective effects of anesthetics on in vitro systems composed of both lipid and protein have been interpreted as meaning that the sites of action are proteins rather than lipids (Franks and Lieb, 1991; Jones and Harrison, 1993). However, the glycerol "backbone" of phospholipids contains a chiral carbon atom and the lipids of nerve plasma membranes include, typically, 40 mol % cholesterol, a steroid with several chiral carbons. Thus it is conceivable that stereoselective effects could be mediated through membrane lipids (Alifimoff et al., 1993). Indeed, two enantiomers of Δ^1-tetrahydrocannabinol have been shown to differentially affect the fluidity of phosphatidylcholine/cholesterol bilayers (Lawrence and Gill, 1975). Although the optical isomers of isoflurane have been shown to produce identical depressions of the chain-melting phase transition temperature (a measure of bilayer disruption) in cholesterol-free bilayers (Franks and Lieb, 1991), no direct measurements of the partition coefficients of isoflurane enantiomers into lipid bilayers (either with or without cholesterol) have been reported. We describe here a novel gas chromatographic method for measuring the partitioning of

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volatile anesthetic enantiomers into cholesterol-free and cholesterol-containing bilayers. The method makes use of a chiral capillary column which can resolve the optical isomers, thus allowing the quantity of each isomer present in the bilayer to be determined. This technique has the advantage of not requiring optically pure volatile anesthetics, allowing the determination of the partition coefficients of the individual enantiomers to be made using only the commercially available racemic mixtures.

MATERIALS AND METHODS

Chemicals

L-α-Phosphatidylcholine and L-α-phosphatidic acid (both from egg yolk), NaCl, Tris-HCl, and sodium azide were obtained from Sigma Chemical Company Ltd. (Poole, Dorset, UK). Cholesterol was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). *n*-Heptane (HPLC grade) was obtained from Aldrich Chemical Company Ltd. (Gillingham, Dorset, UK). Chloroform (Analar grade) was purchased from BDH Ltd. (Poole). Racemic isoflurane was obtained from Abbott Laboratories Ltd. (Queenborough, Kent, UK). Optically pure enantiomers of isoflurane were kindly given to us by the BOC Group Inc. (Murray Hill, NJ).

Preparation of lipid suspensions

Phosphatidylcholine (supplied as a chloroform/methanol solution) was dried down under a gentle stream of nitrogen, followed by rotary evaporation under vacuum for 3.5 h. The dry phosphatidylcholine was weighed and dissolved in chloroform. Appropriate amounts of phosphatidic acid and cholesterol were then weighed out and separately dissolved in chloroform. The chloroform solutions were combined and the resulting mixture dried down as described above, pumping under vacuum for ~40 h to remove all traces of chloroform (as indicated by the lack of any chloroform peak on the gas chromatograph). The dried lipid mixture was suspended in buffer to give a suspension of concentration ~10 mg lipid/ml. The buffer solution used throughout these experiments was: 150 mM NaCl, 0.1 wt % sodium azide, and 10 mM Tris-HCl (titrated to pH 7.4 with NaOH). Lipid volumes were calculated from the exact lipid concentrations using a density of 1.014 g/cm^3 (Johnson and Buttress, 1973). The lipid solution was briefly sonicated (for 20 s) in a bath sonicator to aid dispersion.

The Wishnia cell

The partition measurements were made using a Wishnia cell (Fig. 1) which was specially constructed by Sidney Simon (based on a design by Arnold Wishnia). The Wishnia cell consists of a glass jacket containing four separate chambers interconnected only through the gas phase. The temperature of the Wishnia cell could be regulated by circulation of water through the water jacket, although we did not use this facility, since our experiments were carried out in a temperature-controlled room (21 ± 1°C). Samples of either lipid suspension or buffer (1 ml) were added to each of the four chambers, which were then sealed with rubber stoppers. A small volume (~2 μl) of neat liquid racemic isoflurane was added to the gas phase through the entry port, which was then also sealed. The Wishnia cell was rotated (~25 rpm) horizontally about its long axis so that the polytetrafluoroethylene (PTFE) ball rolled back and forth along a closely fitting glass tube, thus mixing and circulating the vapor phase between the four chambers. The cell was rotated for 45 min, by which time the vapor and aqueous phases had come to equilibrium (data not shown). The concentrations of each of the optical isomers in the aqueous and lipid phases were determined by gas chromatography, as described below.

Heptane extraction

The contents (1 ml of either lipid suspension or buffer) of the four chambers in the Wishnia cell were transferred to four capped (Bakelite screw caps with foil-faced liners) 2-ml glass vials, each containing 0.25 ml of *n*-heptane. The vials were vortexed for 2 min and left to equilibrate at room temperature (21 ± 1°C) for 45 min. The vials containing lipids were then centrifuged (5 min at ~1800 × *g*) to separate the heptane and aqueous phases. Loss of isoflurane from these vials was assessed by gas chromatography and found not to be significant during the time course of a typical extraction procedure (~100 min). This procedure resulted in ~86% of the isoflurane being extracted into the heptane phase.

Gas chromatography

A 2-μl sample of the heptane phase was injected into the gas chromatograph (model 8600, split/splitless injector, electron capture detector, Perkin-Elmer Ltd., Beaconsfield, Bucks, UK). To separate the isoflurane enantiomers, we used an 80-m long Chiraldex G-TA capillary column (Advanced Separation Technologies Inc., Whippany, NJ) having an internal diameter of 0.25 mm. The gas chromatographic parameters were: split ratio 100:1, injector temperature 250°C, detector temperature 350°C, and oven temperature 60°C

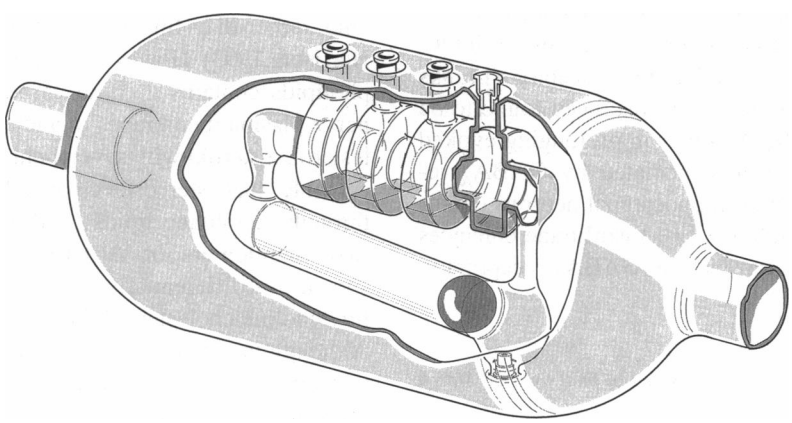

FIGURE 1 The Wishnia cell used for the partition coefficient determinations. The cell consists of four chambers (one shown in cross-section) interconnected through the gas phase and enclosed by a glass jacket (for thermal regulation). Each chamber was loaded with 1 ml of lipid suspension or buffer such that the level of the liquid was below the tube connecting the chambers. A small amount of neat isoflurane, which rapidly evaporated into the gas phase, was added through the entry port (shown at bottom). As the whole cell was rotated, the PTFE ball (lower right) moved back and forth along a closely fitting tube, mixing and circulating the gas phase throughout the chambers.

(isothermal). Peak areas were computed by a built-in microprocessor. The two enantiomers of isoflurane were clearly resolved [see also Meinwald et al. (1991)], as shown in Fig. 2; the peak assignments were made by injecting optically pure S(+) and R(−) isoflurane. The system was calibrated using aqueous solutions of known (racemic) isoflurane concentration (followed by the usual heptane extraction), and it can be seen from Fig. 3 that the areas of the two enantiomer peaks in the chromatogram were equal to one another and proportional to the concentration of racemic isoflurane.

Calculation of partition coefficient ratios

The molar partition coefficient, K, between lipid and buffer is defined as:

$$K \equiv C_{lip}/C_{buf}, \tag{1}$$

where C_{lip} and C_{buf} are the molar concentrations of anesthetic in the lipid membranes and buffer, respectively. If C_{sus} is the molar concentration of anesthetic in the lipid suspension and V_{sus}, V_{buf}, and V_{lip} are the total volumes of lipid suspension, buffer, and lipid membranes, respectively, the conservation equation for anesthetic is:

$$C_{sus}V_{sus}/C_{buf}V_{buf} + C_{lip}V_{lip}. \tag{2}$$

It follows from Eqs. 1 and 2 that

$$K = (C_{sus}V_{sus} - C_{buf}V_{buf})/(C_{buf}V_{lip}). \tag{3}$$

Because the ratio of the isomer concentrations could be determined much more accurately than the absolute concentrations, the most precise estimate of the degree of stereoselective partitioning can be obtained from the ratio of the partition coefficients for the S(+) and R(−) isomers. This can be written as

$$\frac{K^+}{K^-} = \frac{\{C^+_{sus}/C^+_{buf}\}V_{sus} - V_{buf}}{\{C^-_{sus}/C^-_{buf}\}V_{sus} - V_{buf}}, \tag{4}$$

where the plus and minus superscripts refer to the S(+) and R(−) isomers, respectively. Thus it is only the ratio of suspension to buffer anesthetic isomer concentrations, and not their absolute values, which determine the ratio K^+/K^- of partition coefficients. Since anesthetic isomer concentration is proportional to peak area on the chromatogram (Fig. 3), these concentration ratios can be estimated from the ratios of the relevant peak areas on the chromatogram. Furthermore, since water provides an achiral environment, the buffer concentrations C^+_{buf} and C^-_{buf} of the two enantiomers would be expected to be equal on theoretical grounds and were indeed found to be equal experimentally (Fig. 3). Therefore, for estimating the concentration ratios in Eq. 4, we averaged the buffer peak areas for the two isomers to obtain a more accurate estimate. The equation used to estimate the ratio of partition coefficients was thus:

$$\frac{K^+}{K^-} = \frac{\{A^+_{sus}/\langle A_{buf}\rangle\}V_{sus} - V_{buf}}{\{A^-_{sus}/\langle A_{buf}\rangle\}V_{sus} - V_{buf}}, \tag{5}$$

where $\langle A_{buf}\rangle$ was the mean buffer area on the chromatogram and A^+_{sus} and A^-_{sus} were the lipid suspension peak areas for the S(+) and R(−) isomers, respectively.

In each equilibration run, two of the chambers in the Wishnia cell contained buffer and two contained lipid suspension. Each run produced data for two determinations of the ratio of partition coefficients.

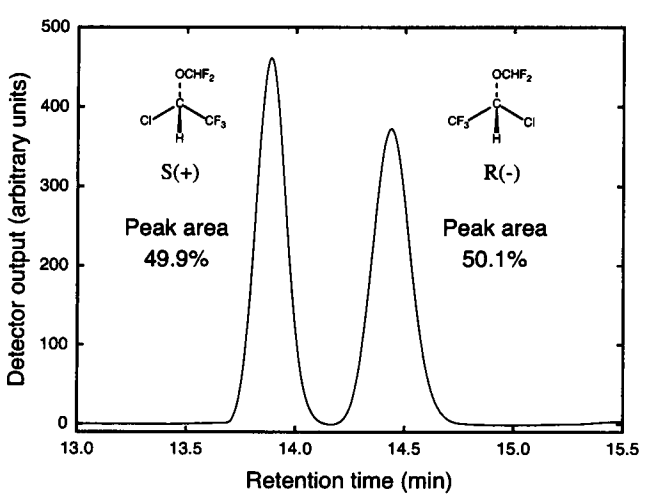

FIGURE 2 A trace from the gas chromatograph showing the resolution of racemic isoflurane into its two optical isomers. Note that, although the peak heights differ, their areas are essentially identical.

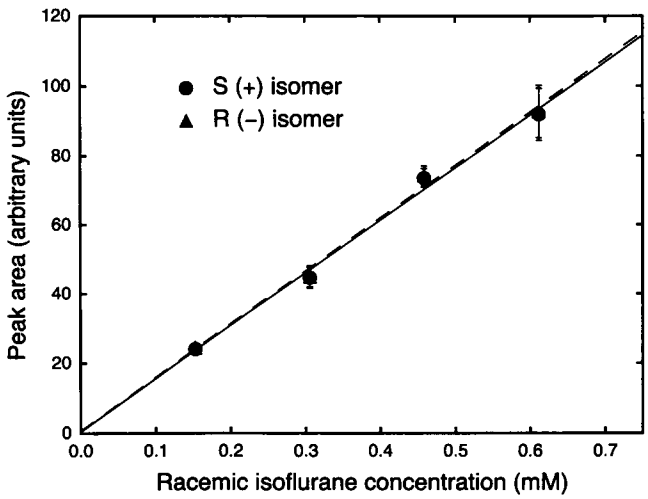

FIGURE 3 The peak areas in the chromatograms were proportional to the concentration of isoflurane in water. Data for the S(+) and R(−) isomers are represented by the symbols ● and ▲, respectively. The points are means of duplicate measurements. The error bars are standard errors and where not shown were smaller than the symbols. The lines are least squares regressions; the full line is for the S(+) isomer and the dashed line is for the R(−) isomer.

RESULTS

The distribution of the optical isomers of isoflurane in lipid bilayer suspensions and in buffer is shown in Fig. 4. In both cholesterol-free (Fig. 4 *A*) and cholesterol-containing (Fig. 4 *B*) bilayer suspensions, and also in buffer, there was little difference in the amount of each isomer present. The ratios K^+/K^- of the S(+) to R(−) isoflurane partition coefficients were found to be very close to unity for both types of bilayer (Table 1). The ratios (mean ± SEM) were 1.011 ± 0.002 for bilayers of PC/PA (mole ratio 96:4) and 1.018 ± 0.010 for bilayers of PC/CHOL/PA (mole ratios 56:40:4). These results show that the lipid solubilities of the optical isomers of the volatile anesthetic isoflurane were essentially identical. This was true in both cholesterol-free and cholesterol-containing lipid bilayers.

The absolute values (mean ± SEM) of the molar partition coefficients for isoflurane were (Table 1) 165 ± 10 and 49 ± 4 for cholesterol-free and cholesterol-containing lipid bilayers, respectively.

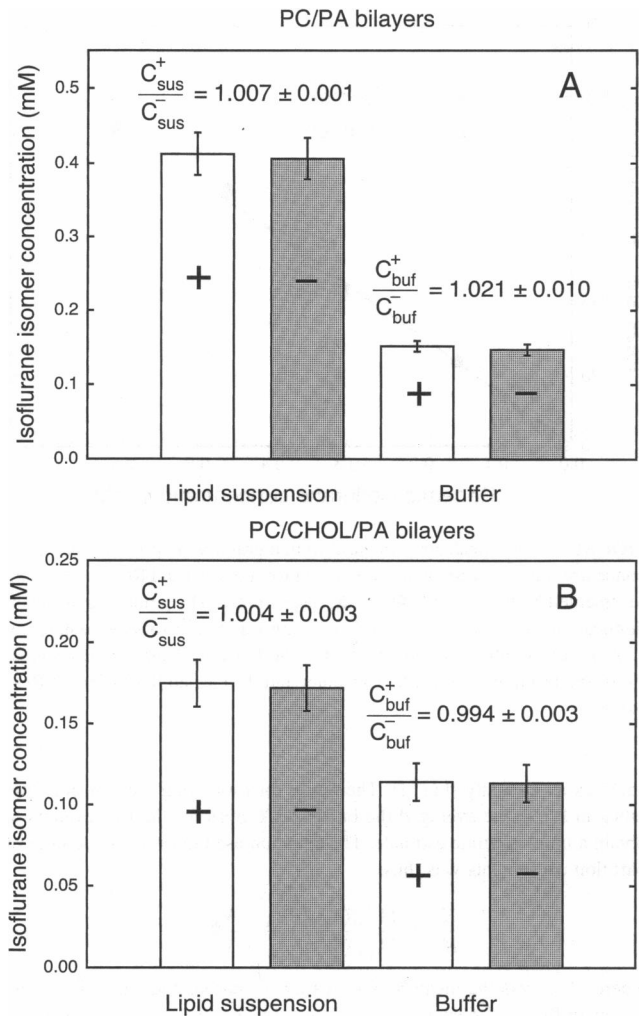

FIGURE 4 The S(+) and R(−) isomers of isoflurane do not show stereoselective partitioning into lipid bilayers. (*A*) Data for PC/PA bilayers (mole ratio 96:4). (*B*) Data for PC/CHOL/PA bilayers (mole ratios 56:40:4). The ratios of the concentrations of the S(+) isomer to the R(−) isomer are given for both the lipid bilayer suspensions and for buffer. The concentration ratios were calculated for each of the 9 or 10 determinations in either lipid suspension or buffer. The quoted values are the means ± SEM. Notice that the errors in the ratios of the two isomer concentrations are much less than the errors in the absolute concentrations.

DISCUSSION

Stereoselective binding of drugs to receptors is, of course, commonplace in pharmacology. It is generally thought, however, that such stereoselectivity does not extend to general anesthetics. This is partly due to the influence of traditional "nonspecific" theories of anesthetic action (Dluzewski et al., 1983; Miller, 1985). However, it is also due to the fact that the potency ratios observed in vivo between anesthetic enantiomers (Ryder et al., 1978; Andrews and Mark, 1982; Richter and Holtman, 1982; White et al., 1985; Harris et al., 1992) are usually found to be relatively small (<4), and it has sometimes been difficult to completely rule out pharmacokinetic explanations.

Studies of the effects of anesthetic enantiomers in vitro are more clear-cut. There is now unambiguous evidence that barbiturates (Huang and Barker, 1980), ketamine (Lodge et al., 1982; Zeilhofer et al., 1992), and even relatively simple agents such as isoflurane (Franks and Lieb, 1991; Jones and Harrison, 1993; Moody et al., 1993) and 2-butanol (Alifimoff et al., 1993) can show stereoselective effects on neuronal ion channels. The question then arises, at the molecular level, as to how this stereoselectivity comes about. Is it due to stereoselective interactions with proteins, lipids, or both? The stereoselective actions of the inhalational anesthetic isoflurane have been interpreted in terms of direct protein binding (Franks and Lieb, 1991; Jones and Harrison, 1993), but it has not been ruled out that the relatively small potency differences observed could be accounted for in terms of differential solubility in a lipid bilayer (Alifimoff et al., 1993), particularly one containing a high concentration of the chiral steroid cholesterol. In this paper we have specifically addressed this question.

The method we have used gives partition coefficients for isoflurane (Table 1) which are in good agreement with those obtained previously for the same, or similar, lipid bilayers. A direct comparison can be made for PC/PA bilayers, where our value of 165 ± 10 is close to the value of 147 (no error given), which can be calculated from the data in Table 1 of Smith et al. (1981) for the same lipid mixture. The substantially reduced partitioning of isoflurane into cholesterol-containing bilayers (Table 1) is also consistent with previous work (Smith et al., 1981).

The results of our experiments with the optical isomers of isoflurane were straightforward. We found (Table 1 and Fig. 4) that the solubility of isoflurane in bulk lipid bilayers is not stereoselective. There was essentially no difference in the lipid bilayer/buffer partition coefficients of the S(+) and the R(−) isoflurane isomers. (The small differences we did observe, of the order of 1%, are probably due to systematic errors.) This was true for both cholesterol-free (Fig. 4 *A*) and cholesterol-containing (Fig. 4 *B*) bilayers. Had the bilayers displayed any stereoselectivity, it might have been anticipated that this would have occurred at low, rather than high, anesthetic levels. For this reason we used very low free aqueous isoflurane concentrations (Table 1), close to the average general anesthetic EC_{50} concentration of 0.32 mM isoflurane for mammals (Franks and Lieb, 1993) and 0.29 mM isoflurane for tadpoles (Firestone et al., 1986).

Our result of negligible stereoselectivity for partitioning of isoflurane into PC/PA bilayers without cholesterol is consistent with previous results showing equal depressions by S(+) and R(−) isoflurane of the main chain-melting phase transition temperature of dipalmitoyl phosphatidylcholine bilayers (Franks and Lieb, 1991). They are also consistent with the negative results of an early halothane electron spin resonance study (Kendig et al., 1973) on egg phosphatidylcholine bilayers. However, this latter study used mixtures of halothane enantiomers with very poor optical purity: the purest preparation had an enantiomer ratio of only 75%:25% (compared with the ~99%:1% ratios of isoflurane enantiomers now available).

TABLE 1 Molar partition coefficients K between lipid bilayers and buffer for isoflurane and ratios of the partition coefficients K^+/K^- for optical isomers

Bilayer composition	K	K^+/K^-	Isoflurane concentration* (μM)	Number of observations
PC/PA	165 ± 10	1.011 ± 0.002	290 ± 12	9
PC/CHOL/PA	49 ± 4	1.018 ± 0.010	230 ± 16	10

Values are given as means ± SEM.

* Free aqueous racemic isoflurane concentration.

In the absence of any appreciable stereoselective partitioning into lipid bilayers, the stereoselective actions of isoflurane on neuronal ion channels (see Introduction) can most plausibly be accounted for in terms of the anesthetic binding directly to protein molecules, either the channels themselves or regulatory proteins. It is possible, of course, that our measurements of partitioning into bulk lipid bilayers did not show up chiral partitioning into a bilayer region (such as the chiral glycerol backbone region of PC) that could be postulated to be of particular importance for ion channel function. However, at the level of precision of our measurements (1–2%), such putative chiral binding sites in the bilayer are not evident. On the other hand, the stereoselective effects of isoflurane observed with anesthetic-sensitive ion channels are consistent with direct effects on proteins. For example, it has recently been reported that the binding constant of S(+) isoflurane is 50% greater than that of R(−) isoflurane for binding to the soluble protein bovine serum albumin (Eckenhoff and Shuman, 1993). This is the same as the ratio of binding constants derived from isoflurane enantiomer dose-response data for the inhibition of a neuronal nicotinic acetylcholine receptor channel (Franks and Lieb, 1991).

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