Entropy-Driven Interactions of Anesthetics with Membrane Proteins†

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ABSTRACT: Thermodynamic analysis of anesthetic effects on Ca^{2+}-ATPase activity was performed to evaluate the feasibility of anesthetic binding and gain insight into the molecular events underlying the anesthetic−enzyme interactions. The Ca^{2+}-ATPases, integral membrane proteins vital in cellular Ca^{2+} regulation, are suitable models for investigation of the mechanism of anesthetic action on membrane proteins that are targeted by the anesthetics. Ca^{2+}-ATPase of plasma membrane, PMCA, and SERCA1 in the intracellular sarcoplasmic reticulum membrane were used to study two general anesthetics: halothane, a halogenated two-carbon alkane; and propofol, an intravenous, strongly lipophilic-substituted phenol. Interactions of both anesthetics result in a negative Gibbs free energy change, which in both enzymes is more favorable for the more lipophilic propofol than halothane. Temperature dependence (more negative change in Gibbs free energy at increased temperature) is in agreement with predominantly nonpolar interactions. The interactions are entropy-driven, characterized by positive enthalpy which is overcompensated by positive entropy changes. This is in contrast to the reported in literature enthalpy-driven anesthetic binding to soluble proteins. The possible contributions to the observed positive entropy change are discussed including displacement of ordered water molecules by anesthetic binding in nonpolar cavities in the membrane proteins and subtle structural rearrangements.

Membrane proteins have been considered as the most likely pharmacological targets for volatile anesthetics (VA),[1] a clinically important group of general anesthetics. The occurrence of interactions between VA and multiple target proteins *in vivo* is expected to result in the complex phenomenon of general anesthesia and its numerous side effects. Various functional effects of VA have been reported for several membrane proteins studied *in vitro*; however, the molecular mechanism of anesthetic action on any of them is not known. We have recently established a connection between the *in vitro* effects of VA on the activity and changes in spectroscopic properties of an integral plasma membrane protein, Ca^{2+}-ATPase (PMCA), that points to VA effects on enzyme conformation (Lopez & Kosk-Kosicka, 1995). The observed correlation is in agreement with VA binding in the protein that interferes with the conformational transition which the enzyme normally undergoes in its catalytic cycle. We have proposed that such binding is responsible for the observed inhibition of enzymatic function (Lopez & Kosk-Kosicka, 1995). Recently, using direct labeling with [^{14}C]halothane of SERCA1, we have demonstrated that the anesthetic binds saturably to the sarcoplasmic reticulum (SR) membranes, and 40−50% label incorporates directly in the Ca^{2+}-ATPase protein (Kosk-Kosicka et al., 1997). Binding of small ligands, including a gaseous anesthetic xenon, in interior nonpolar sites (cavities) of protein which affects the internal motions and substates was demonstrated for metmyoglobin by X-ray diffraction and NMR data (Schoenborn & Fetherstone, 1967; Tilton et al., 1984). Various techniques have then provided ample evidence for selective VA binding in other soluble proteins including the best studied firefly luciferase where VA compete with the natural substrate luciferin for the binding in a nonpolar pocket of the substrate binding site, and serum albumin (Ueda & Kamaya, 1973; Franks & Lieb, 1984; Dubois & Evers, 1992; El-Maghrabi & Eckenhoff, 1993; Eckenhoff & Shuman, 1993). While anesthetics' action on these proteins is easier to study than on membrane proteins, their characteristics could differ. Thermodynamic analysis of anesthetic−protein interactions has been limited to only soluble proteins, showing that they are enthalpy-driven (Ueda & Kamaya, 1973; Ewing & Maestas, 1970). We have presently characterized thermodynamically the anesthetic interactions with Ca^{2+}-ATPases. Two enzyme preparations were compared: the purified, solubilized plasma membrane PMCA and SERCA1 in the native environment of intracellular SR membranes. When studied *in vitro* at clinical halothane concentrations, SERCA1 is activated and then inhibited at higher concentrations. PMCA is more sensitive than SERCA1 to the inhibitory effects of various VA (Lopez & Kosk-Kosicka, 1995). For comparison, we have included a different (nonvolatile, intravenous) general anesthetic, propofol, recently introduced in clinical anesthesia. Two models of interactions of small ligands (anesthetics) with proteins have been considered. The first, widely used in literature, gives binding characteristics for the hypothetical standard state in which ligand concentration is 1 M (Dickinson et al., 1993; Raffa & Porreca, 1989; Morton et al., 1995). The second takes into account ligand and protein concentrations (Schellman, 1987; Makhatadze & Privalov, 1992). Both analyses yield thermodynamic

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[1] Abbreviations: PMCA, plasma membrane Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; SERCA1, skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase; VA, volatile anesthetics; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; Me_2SO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide; NMF, *N*-methylformamide.

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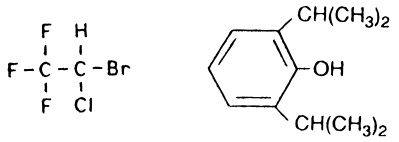

halothane propofol

FIGURE 1: Chemical structures of anesthetics used in the study.

parameters that characterize binding of the anesthetics to the ATPases as thermodynamically favorable and, in contrast to the soluble proteins, entropy-driven processes.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine (P5763) and CNBr-activated Sepharose 4B were purchased from Sigma; octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was from Nikko (Tokyo, Japan). Coupling of bovine calmodulin to Sepharose was performed as described previously (Kosk-Kosicka & Bzdega, 1988). Thymol-free halothane was obtained from Halocarbon Laboratories (River Edge, NJ). 2,6-Diisopropylphenol (propofol), *N*,*N*-dimethylformamide (DMF), and *N*-methylformamide (NMF) were purchased from Aldrich; dimethyl sulfoxide (Me_2SO) was from J. T. Baker. Structures of propofol and halothane are shown in Figure 1.

The methods used for preparation of erythrocyte membranes and subsequent purification of Ca^{2+}-ATPase (PMCA) and determination of protein and Ca^{2+} concentrations were as described previously (Kosk-Kosicka et al., 1986; Kosk-Kosicka & Bzdega, 1988). Briefly, the enzyme was purified from human erythrocyte membrane ghosts by calmodulin affinity column chromatography in the presence of the nonionic detergent $C_{12}E_8$. Phosphatidylcholine was added to the eluted enzyme at the final concentration of 0.02%. Protein concentration was measured by Bio-Rad protein micro-assay based on the Bradford dye-binding procedure, using BSA as a standard. Total calcium was measured by atomic absorption, and free Ca^{2+} concentrations were calculated based on the constants given by Schwartzenbach et al. (1957). Sarcoplasmic reticulum (SR) was prepared from rabbit skeletal muscle in the laboratory of Dr. Inesi (Eletr & Inesi, 1972).

Ca^{2+}-ATPase Activity Assay. Ca^{2+}-ATPase activity was determined by measurements of inorganic phosphate production, generally as described previously (Kosk-Kosicka & Bzdega, 1988). The activity assay was performed on the purified PMCA at a 30 nM concentration in a reaction mixture containing 50 mM Tris−maleate, pH 7.4, 120 mM KCl, 8 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, and $CaCl_2$ to yield 17 μM free Ca^{2+}. The concentration of $C_{12}E_8$ was kept constant at 150 μM. For activity assay of the Ca^{2+}-ATPase in SR, the divalent cation ionophore A23187 (10 μM) was included instead of $C_{12}E_8$. All assays were performed in sealed 0.65 mL polypropylene tubes in a total reaction volume of 0.33 mL. Following the addition of all reagents and protein, halothane was delivered to the tube in an air-tight Hamilton syringe, and the reaction was started with 3 mM ATP; it was carried out for up to 30 min for PMCA and up to 15 min for SR Ca^{2+}-ATPase. The reaction was terminated with ammonium molybdate/metavanadate at individual times. Steady-state velocities were obtained from plots of P_i production which were linear with time. Halothane was delivered to the assay either as saturated aqueous solutions (of the same composition as the reaction mixture) or dissolved in DMF, NMF, or Me_2SO. Final concentration of the solvents during the assay was 2%. Halothane solutions were prepared daily from the stock of pure volatile anesthetic under nitrogen gas at room temperature. Halothane concentration in the reaction mixture during the assay was calculated and confirmed by gas chromatography. For halothane delivered from the saturated aqueous solution, the corrected values are used which are 10-fold higher than indicated in previous publications from this laboratory. Propofol was dissolved in Me_2SO.

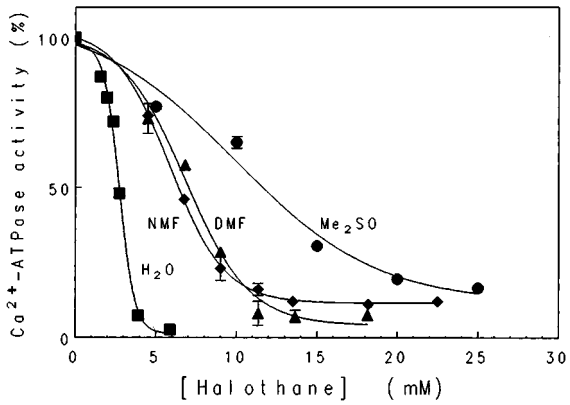

FIGURE 2: Inhibition of PMCA Ca^{2+}-ATPase activity by halothane in different solutes: water (■); NMF (◆); DMF (▲); Me_2SO (●). The Ca^{2+}-ATPase activity was assayed as described under Materials and Methods. The reaction mixture contained 50 mM Tris−maleate, pH 7.4, 120 mM KCl, 8 mM $MgCl_2$, 17.5 μM free Ca^{2+}, 150 μM $C_{12}E_8$, 1 mM EGTA, and 3 mM ATP. Protein concentration was 30 nM (4 μg/mL). The assays were performed at 25 °C. The specific activity (100%) was 220 ± 9 μmol of P_i (mg of protein)$^{-1}$ h^{-1}.

Anesthetic Molecule Volume Estimation. The halothane and propofol molecules were built and minimized using a commercial program (Quanta Molecular Simulations Inc). Default parameter set was used.

RESULTS

Inhibition of Ca^{2+}-ATPase Activity by Halothane in Different Solutes. The effect of halothane on Ca^{2+}-ATPase activity was investigated on two different Ca^{2+}-ATPases: a plasma membrane enzyme, PMCA, which was purified from red cells and an intracellular membrane enzyme, SERCA1, which was studied in the SR membrane environment. Halothane was delivered to the reaction mixture either as a saturated aqueous solution, which is the preferred method for *in vitro* studies, or in one of the three solutes specified in Figure 2. Dimethyl sulfoxide (Me_2SO), methylformamide (NMF), and dimethylformamide (DMF) were used for comparison because propofol, the other anesthetic studied, needs to be dissolved in Me_2SO and because DMF was used by other authors (Karon & Thomas, 1993). None of the solutes at the concentrations used affects enzyme activity significantly in the absence of halothane (Kosk-Kosicka et al., 1994; Kosk-Kosicka & Roszczynska, 1994). Figures 2 and 3A show that the Ca^{2+}-ATPase activity of both enzymes is inhibited by halothane in a dose-dependent manner. The extent of halothane-induced inhibition depends strongly on the solute in which the anesthetic is delivered to the enzyme. The concentration of halothane at which PMCA activity is inhibited half-maximally (k_{Hal}) at 25 °C varies from 2.6−2.7 mM halothane in the aqueous solution to 6−7 mM in

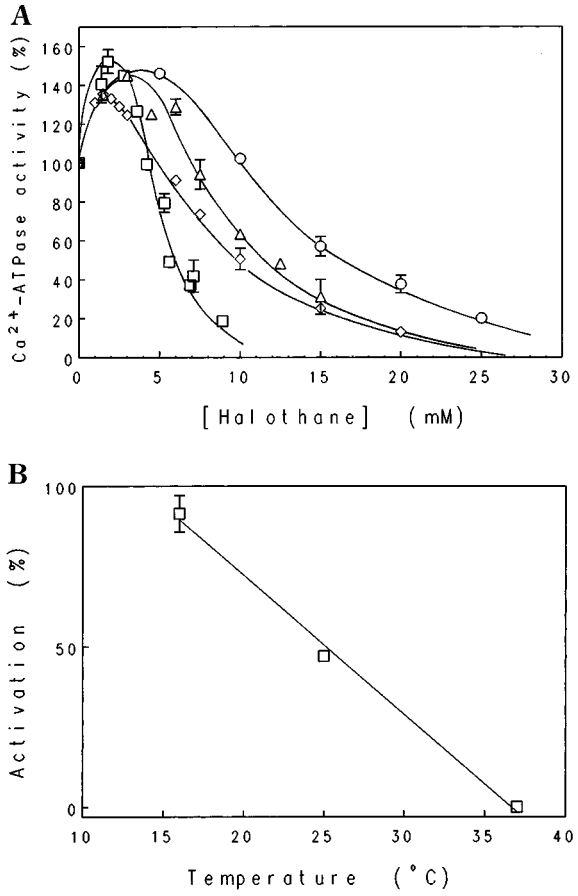

FIGURE 3: (A) Inhibition of SERCA1 Ca^{2+}-ATPase activity by halothane in different solutes. The experimental conditions were as described in Figure 1, except $C_{12}E_8$ was omitted and instead 10 μM ionophore A23187 was included in the reaction mixture. The concentration of the SR protein was 2.7 μg/mL. The specific activity (100%) was 100 μmol of P_i (mg of protein)$^{-1}$ h^{-1}. Appropriate (see Figure 2) open symbols were used. (B) Correlation between the extent of SERCA1 activation (observed at lower halothane concentrations) and the assay temperature (16, 25, and 37 °C). Halothane concentration was 1.8 mM and delivered from aqueous solution.

NMF, 7−8 mM in DMF, and 11−12 mM halothane in Me_2SO (Figure 2). There is no significant difference in halothane solubility in the three organic solvents, as determined by gas chromatography.

The difference in sensitivity of Ca^{2+}-ATPase activity to halothane in diverse solutes is also observed for SERCA1 (Figure 3A). The order of inhibition by halothane is the same, with k_{Hal} at 25 °C of 6−7 mM in "water", 9−11 mM in NMF, 11−12 mM in DMF, and 16−17 mM in Me_2SO. However, in contrast to PMCA, in the initial phase SERCA1 is activated by halothane in all four solutes. The activation shows a strong linear correlation when analyzed as a function of temperature for halothane delivered from the saturated aqueous solution (Figure 3B).

Anesthetic−Enzyme Interaction: Thermodynamic Analysis. Anesthetic-induced inhibition of Ca^{2+}-ATPase activity was analyzed in thermodynamic terms as resulting from anesthetic-enzyme interaction. Two different models were used. Model 1 (eqs 1−3) provides standard thermodynamic parameters. This model has been widely used in literature to express the energetics of binding; it gives binding characteristics for the standard state in which the ligand concentration is 1 M [for example, see Dickinson et al. (1993) in the analysis for firefly luciferase]. In practice, the concentrations of free ligands are much lower. The standard thermodynamic parameters are considered a measure of the intrinsic binding parameters. To obtain real energetics of anesthetic binding to the proteins, we have used model 2 (eqs 4−6). In this model, all thermodynamic parameters (free energy, enthalpy, and entropy) depend explicitly on ligand (anesthetic) concentration [for example, see Makhatadze and Privalov (1992) in the analysis of urea binding to proteins], so each experimental ligand concentration is considered the standard state. Model 2 is more realistic than model 1 because it uses as standard state experimentally achievable ligand concentrations as opposed to the assumed hypothetical 1 M standard state. Application of both models provides the opportunity to evaluate the importance of the thermodynamic contributions from independent calculations.

To characterize the halothane-induced inhibition of Ca^{2+}-ATPase activity, the thermodynamic analysis was performed for halothane delivered in aqueous solution and in Me_2SO, two solutes for which the biggest difference in k_{Hal} is observed (Figures 2 and 3A).

(A) Model 1. The analysis was done using eqs 1−3 (Creighton, 1993). The Gibbs free energy ($\Delta G_{solute\rightarrow enzyme}$), the enthalpy ($\Delta H_{solute\rightarrow enzyme}$), and the entropy ($\Delta S_{solute\rightarrow enzyme}$) for the transfer of anesthetic molecule from aqueous solution to the Ca^{2+}-ATPase are defined by

$$\Delta G_{solute\rightarrow enzyme} = -RT \ln (1/k_{Anesth}) \quad (1)$$

$$\Delta H_{solute\rightarrow enzyme} = \mathrm{d}(\Delta G_{solute\rightarrow enzyme}/T)/\mathrm{d}(1/T) = -R\, \mathrm{d} \ln (1/k_{Anesth})/\mathrm{d}(1/T) \quad (2)$$

$$\Delta S_{solute\rightarrow enzyme} = (\Delta H_{solute\rightarrow enzyme} - \Delta G_{solute\rightarrow enzyme})/T \quad (3)$$

where R is the universal gas constant, T is the absolute temperature, and k_{Anesth} is the dissociation constant (k_{Anesth} equals k_{Hal} or k_{Prop}, appropriately). The k_{Anesth} is an estimate of the binding constant. This assumption may or may not be true. However, it is in agreement with the results of photoaffinity labeling of SR membranes with [^{14}C]halothane which produced a binding constant for SERCA1 that agrees very well with the concentration at which halothane half-maximally activates this enzyme (Kosk-Kosicka et al., 1997). For technical reasons, the binding could be studied only for the activatory phase of the halothane effect on SERCA1. For PMCA, we have shown that the inhibition correlates well with the elimination of the crucial conformational change induced by Ca^{2+} binding in the initial step of the enzymatic cycle (Lopez & Kosk-Kosicka, 1995). As a first approximation, in an attempt to simplify the mathematical treatment, we are considering the inhibition constants for the two enzymes as the dissociation constants for the anesthetic−protein interaction. The analysis assumes that the enthalpy of anesthetic−enzyme interaction is temperature-independent (van't Hoff enthalpy) (Creighton, 1993). $\Delta H_{solute\rightarrow enzyme}$ was obtained from the slope of ln ($1/k_{Anest}$) *versus* $1/T$ (Figure 4A,B). Strong linear correlation of ln ($1/k_{Anest}$) *versus* $1/T$ (correlation factor equal to 0.98−0.99) indicates that measurements performed at three temperatures adequately describe the temperature behavior of the inhibition process.

Comparison of the thermodynamic parameters for the two enzymes reveals both differences and similarities (Table 1). First, k_{Anesth} is about 2.8 times higher for SERCA1 than for

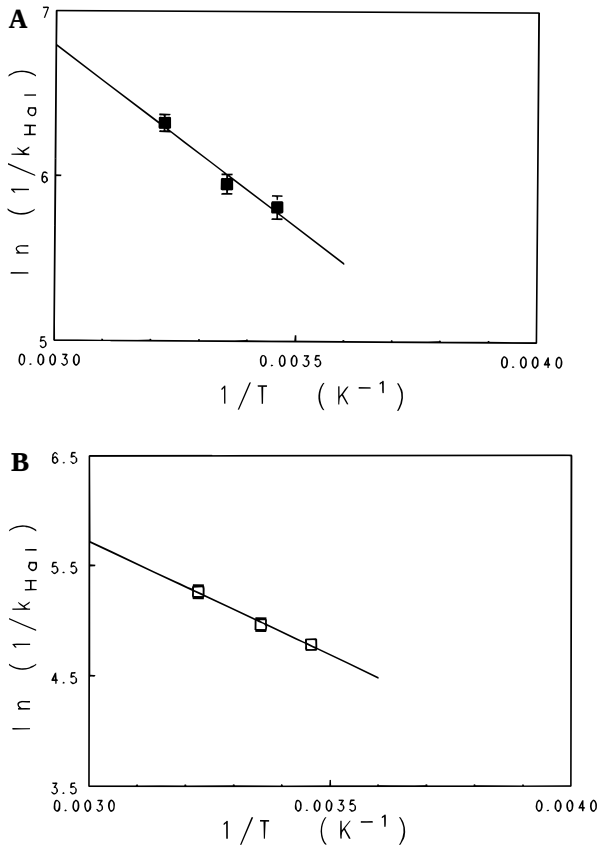

FIGURE 4: van't Hoff plots for halothane inhibition of Ca^{2+}-ATPase activity of PMCA (A) and SERCA1 (B). Halothane was delivered from aqueous solution. The mean inhibition constants are plotted against the reciprocal of the absolute temperature. The error bars are the standard deviation of the mean; where not shown, these are smaller than the symbols. Correlation factors are 0.983 (A) and 0.997 (B).

PMCA. Second, for both enzymes, $\Delta H_{\text{solute}\rightarrow\text{enzyme}}$ and $\Delta S_{\text{solute}\rightarrow\text{enzyme}}$ are positive with values about 15% larger for PMCA than for SERCA1. Third, for both enzymes the entropy term ($-T\Delta S_{\text{solute}\rightarrow\text{enzyme}}$) overcompensates the enthalpic term ($\Delta H_{\text{solute}\rightarrow\text{enzyme}}$), so that the Gibbs energy of the process ($\Delta G_{\text{solute}\rightarrow\text{enzyme}}$) is negative, thermodynamically favorable.

(B) Model 2. The analysis was done according to eqs 4−6 (Makhatadze & Privalov, 1992), with the distinction that we have used dissociation, not association, constants. Assuming the simplest case of independent and equivalent binding sites, the Gibbs energy change ($\Delta G_{\text{binding}}$), the enthalpy change ($\Delta H_{\text{binding}}$), and the entropy change ($\Delta S_{\text{binding}}$) upon binding of one molecule of the anesthetic to the protein are defined by

$$\Delta G_{\text{binding}} = -RT \ln\left(1 + \frac{[\text{anesthetic}]}{k_{\text{Anesth}}}\right) \quad (4)$$

$$\Delta H_{\text{binding}} = \mathrm{d}(\Delta G/T)/\mathrm{d}(1/T) = -R\,\mathrm{d}\ln(1 + [\text{anesth}]/k_{\text{Anesth}})/\mathrm{d}(1/T) \quad (5)$$

$$\Delta S_{\text{binding}} = (\Delta H_{\text{binding}} - \Delta G_{\text{binding}})/T \quad (6)$$

This formulation describes explicitly the difference in energetics of the free and ligated anesthetic without establishing a particular standard state as is necessary in model 1 (Schellman, 1987; Wyman & Gill, 1990). The complete mathematical treatment for calculating ΔH is

$$\Delta H_{\text{binding}} = -R\,\mathrm{d}\ln\left(1 + \frac{[\text{anesth}]}{k_{\text{Anesth}}}\right)/\mathrm{d}(1/T) = \frac{-R}{1 + ([\text{anesth}]/k_{\text{Anesth}})}\frac{\mathrm{d}[1 + ([\text{anesth}]/k_{\text{Anesth}})]}{\mathrm{d}(1/T)} = \frac{-R}{1 + ([\text{anesth}]/k_{\text{Anesth}})}\left(\frac{1}{k_{\text{Anesth}}}\frac{\mathrm{d}[\text{anesth}]}{\mathrm{d}(1/T)} + [\text{anesth}]\frac{\mathrm{d}(1/k_{\text{Anesth}})}{\mathrm{d}(1/T)}\right) = \frac{-R}{1 + ([\text{anesth}]/k_{\text{Anesth}})}\left(\frac{1}{k_{\text{Anesth}}}\frac{\mathrm{d}[\text{anesth}]}{\mathrm{d}(1/T)} + \frac{[\text{anesth}]}{k_{\text{Anesth}}}\frac{\mathrm{d}\ln(1/k_{\text{anesth}})}{\mathrm{d}(1/T)}\right)$$

If we consider that the change in anesthetic concentration with temperature can be ignored then

$$\Delta H_{\text{binding}} = \frac{[\text{anesth}]}{[\text{anesth}] + k_{\text{Anesth}}}\left(\frac{-R\,\mathrm{d}\ln(1/k_{\text{Anesth}})}{\mathrm{d}(1/T)}\right) = \frac{[\text{anesth}]}{[\text{anesth}] + k_{\text{Anesth}}}\Delta H(\text{from model 1})$$

Comparison of the Gibbs free energy change for the two ATPases calculated for increasing halothane concentrations at three temperatures (Figure 5A) shows that (i) $\Delta G_{\text{binding}}$ is negative (*i.e.*, anesthetic binding to the enzymes is a spontaneous event at the pressure and temperatures of the experiments); (ii) it increases as a function of anesthetic concentration (leading to a progressive inhibition of Ca^{2+}-ATPase activity); (iii) at all temperatures, the Gibbs free energy change ($\Delta G_{\text{binding}}$) is 2 times higher for halothane binding to PMCA as compared to SERCA1. The values of $\Delta H_{\text{binding}}$ and $\Delta S_{\text{binding}}$ are positive for both enzymes (see Table 2 for an example of values calculated for one temperature, 25 °C). The values are higher for PMCA than SERCA1 at comparable halothane concentrations.

Thus, the analysis of anesthetic action on the Ca^{2+}-ATPase using either model 1 or model 2 consistently indicates that halothane interaction with the enzyme is an entropy-driven (the entropy term overcompensates the enthalpic term) process.

Analysis of the Interaction of PMCA and SERCA1 with Halothane in Me_2SO. Since propofol (analyzed below) by virtue of its high lipophilicity needed to be dissolved in Me_2SO, we have also performed the thermodynamic analysis for halothane dissolved in Me_2SO such that concentrations of the solute in both assays were comparable.

(A) Model 1. The calculated $\Delta G_{\text{solute}\rightarrow\text{enzyme}}$ values at 25 °C (according to eq 1) for the interaction of halothane dissolved in Me_2SO with PMCA and SERCA1 are −2.6 and −2.4 kcal/mol, respectively. The calculated k_{Hal} are 11.5 (for PMCA) and 16.5 mM (for SERCA1). $\Delta H_{\text{solute}\rightarrow\text{enzyme}}$ and $\Delta S_{\text{solute}\rightarrow\text{enzyme}}$ are positive with values of 6.1 ± 0.3 kcal/mol and 28 ± 1 cal mol^{-1} K^{-1} for SERCA1. The higher k_{Hal} values for anesthetic in Me_2SO, as compared to halothane in saturated aqueous solution, result in a lower change in the Gibbs energy for both enzymes.

Table 1: Intrinsic Thermodynamic Parameters Calculated According to Equations 1−3 (Model 1) for Halothane Interaction with PMCA and SERCA1

	PMCA				SERCA1			
T (°C)	k_{Anesth} (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})	k_{Anesth} (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})
16	3.0			−3.3	8.4			−2.7
25	2.6	4.7 ± 0.9	28 ± 2	−3.5	7.0	4.1 ± 0.3	24 ± 1	−2.9
37	1.8			−4.2	5.2			−3.2

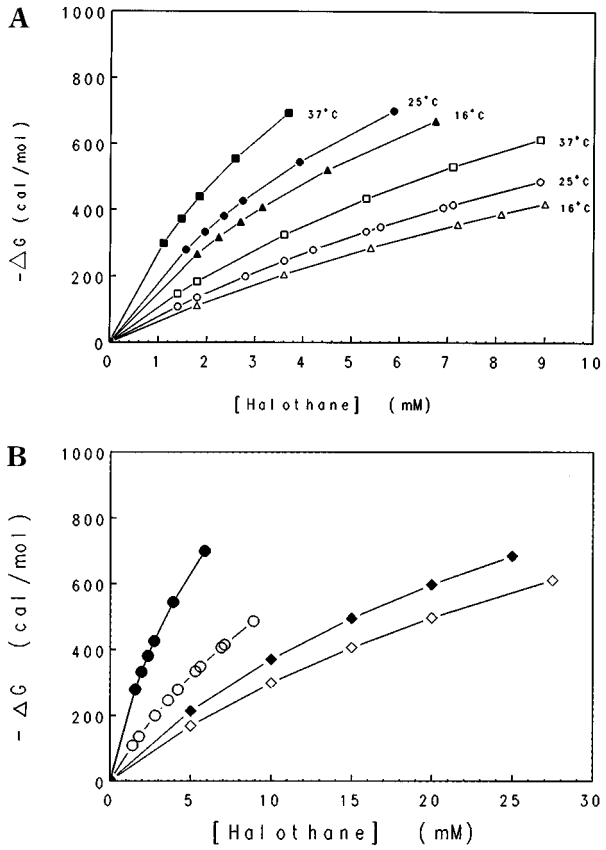

FIGURE 5: (A) Dependence of the change in the free Gibbs energy on halothane concentration. Halothane was delivered from aqueous solution. The experiments were performed for PMCA (filled symbols) and SERCA1 (open symbols) at different temperatures: 16 °C (▲△); 25 °C (●, ○); 37 °C (■, □). (B) Solute dependence of the change in the free Gibbs energy on halothane concentration for PMCA (filled symbols) and SERCA1 (open symbols) at 25 °C. Data for halothane delivered in Me_2SO (◆,◇) are compared to halothane in H_2O [data points as in panel A (●, ○)]. Reaction mixture composition and protein concentration for the two enzymes were as described in Figures 2 and 3, respectively. The ΔG was calculated according to eq 4 (model 2).

(B) Model 2. Comparison of the Gibbs free energy change for binding of halothane in Me_2SO (calculated according eq 4) *versus* halothane in water to the Ca^{2+}-ATPase as a function of anesthetic concentration at 25 °C is shown in Figure 5B. Also in this analysis the interaction of both PMCA and SERCA1 with the anesthetic is thermodynamically less favorable for halothane delivered in Me_2SO.

Analysis of Enzyme Interaction with Propofol. To test the applicability of the two models to the interaction of other general anesthetics with Ca^{2+}-ATPases, we have included in our study propofol, a highly lipophilic intravenous anesthetic. Propofol alters the Ca^{2+}-ATPase activity of both enzymes in a dose-dependent manner similar to halothane (Figure 6A,C). However, the values k_{Prop} are 1−2 orders of magnitude lower than for halothane.

Table 2: Dependence of the Change in Enthalpy and Entropy on Halothane Concentration at 25 °C for PMCA and SERCA1[a]

PMCA			SERCA1		
[halothane] (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	[halothane] (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
0	0	0	0	0	0
1.56	1.76	6.86	1.40	0.68	2.65
1.96	2.02	7.89	1.80	0.84	3.27
2.35	2.23	8.76	2.80	1.17	4.60
2.74	2.41	9.52	3.60	1.39	5.50
3.91	2.82	11.30	4.20	1.54	6.09
5.87	3.26	13.27	5.30	1.77	7.05
			5.60	1.82	7.28
			6.90	2.03	8.19
			7.10	2.06	8.32
			8.90	2.30	9.33

[a] ΔH and ΔS were calculated according to eqs 5 and 6 (model 2). ΔG are as shown in Figure 5A; k_{Hal} equals 2.6 mM for PMCA and 7.0 mM for SERCA1.

*(A) Model 1. A*pplication of eqs 1−3 for characterization of the inhibition of the Ca^{2+}-ATPase activity by propofol showed (Table 3) that similar to halothane the intrinsic thermodynamic parameters $\Delta H_{solute\rightarrow enzyme}$ and $\Delta S_{solute\rightarrow enzyme}$ are positive and $\Delta G_{solute\rightarrow enzyme}$ is negative. In contrast to halothane in water, but similar to halothane in the presence of Me_2SO, the ΔH and ΔS values for PMCA and SERCA1 are alike. The Gibbs energy change is more negative for propofol transfer into both enzymes as compared to halothane.

(B) Application of model 2 (eqs 4−6) to the propofol interaction with the Ca^{2+}-ATPases also yields positive $\Delta H_{binding}$ and $\Delta S_{binding}$ and negative $\Delta G_{binding}$ (Table 4 and Figure 7). Similar to halothane, propofol binding to the Ca^{2+}-ATPase becomes more favorable with increased temperature. As in the analysis using model 1, the change in Gibbs energy in the presence of propofol is more favorable than in the presence of halothane. The difference in ΔG between PMCA and SERCA1 is even less than for halothane in Me_2SO.

DISCUSSION

Our study shows that the two models are applicable to the analysis of general anesthetics' interactions with Ca^{2+}-ATPases. They both support anesthetic binding in the two enzymes and provide the following main characteristics: (i) ΔG is negative under all conditions studied (the temperature range of 16−37 °C and changing anesthetic concentrations), *i.e.*, the interaction is thermodynamically favorable; (ii) ΔS overcompensates ΔH, *i.e.*, the anesthetic−enzyme interaction is entropy-driven; and (iii) temperature dependence (more negative ΔG at increased temperature) is in agreement with nonpolar interactions between the anesthetics and enzymes.

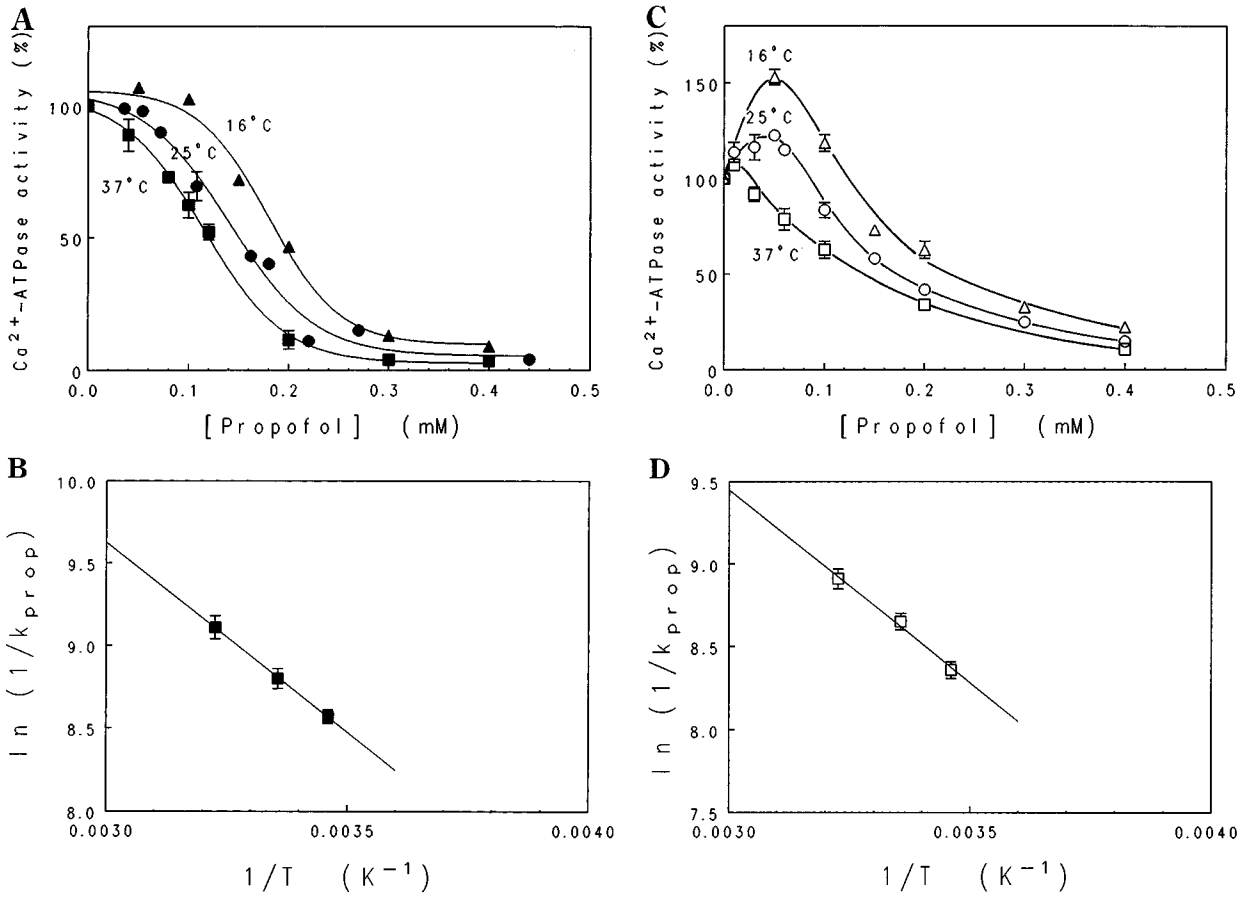

FIGURE 6: Propofol effect on Ca^{2+}-ATPase activity in PMCA (A) and SERCA1 (C) at three temperatures: 16 °C, 25 °C, and 37 °C. Propofol was dissolved in Me_2SO. Reaction mixture composition and protein concentration for the two enzymes were as described in Figures 2 and 3, respectively. Panels B and D show van't Hoff plots for propofol. Correlation factors are 0.999 (B) and 0.995 (D).

Table 3: Intrinsic Thermodynamic Parameters Calculated According to Equations 1−3 (Model 1) for Propofol Interaction with PMCA and SERCA1

	PMCA				SERCA1			
T (°C)	k_{Anesth} (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})	k_{Anesth} (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})
16	0.19			−4.9	0.235			−4.8
25	0.15	4.6 ± 0.1	33 ± 0.5	−5.2	0.175	4.7 ± 0.5	33 ± 1	−5.1
37	0.11			−5.6	0.135			−5.5

Table 4: Dependence of the Change in Enthalpy and Entropy on Propofol Concentration at 25 °C for PMCA and SERCA1[a]

PMCA			SERCA1		
[propofol] (mM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	[propofol] (mM)	ΔH (kcal mol^{-1})	ΔS cal mol^{-1} K^{-1})
0	0	0	0	0	0
0.05	1.16	4.48	0.05	1.04	4.00
0.10	1.85	7.25	0.10	1.71	6.63
0.15	2.31	9.16	0.15	2.17	8.51
0.20	2.64	10.57	0.20	2.51	9.93
0.30	3.08	12.54	0.30	2.97	11.94
0.40	3.36	13.87	0.40	3.27	13.33

[a] ΔH and ΔS were calculated according to eqs 5 and 6 (model 2). ΔG are as shown in Figure 7; k_{Prop} equals 0.150 mM for PMCA and 0.175 mM for SERCA1.

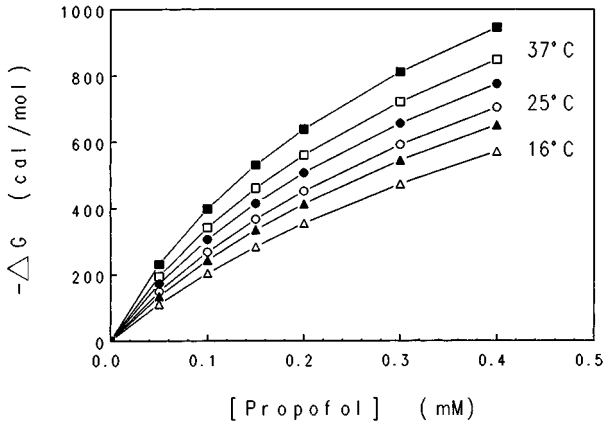

FIGURE 7: Temperature dependence of the change in the free Gibbs energy on propofol concentration [analyzed by eq 4 (model 2)] for PMCA (filled symbols) and SERCA1 (open symbols). Propofol was dissolved in Me_2SO. Experimental conditions and symbols as in Figure 6A,C.

Interactions of Both Anesthetics with Ca^{2+}-ATPases in Contrast to Soluble Proteins Are Entropy-Driven. The intrinsic free energy change for halothane interaction with the Ca^{2+}-ATPase (−3.5 kcal/mol for PMCA and −2.9 kcal/mol for SERCA1 at 25 °C) is in the range of −2.7 to −4.5 kcal/mol reported for interactions of various VA, including halothane, diethyl ether, chloroform, and xenon, with soluble

proteins (Dickinson et al., 1993; Ewing & Maestas, 1970). For example, for the firefly luciferase (experiments performed at 20 °C) ΔG equals −4.5 kcal/mol for halothane and −2.52 kcal/mol for diethyl ether [as recalculated from Dickinson et al. (1993)]. Only model 1 was used in the published thermodynamic analysis of VA binding to soluble proteins.

Our analysis reveals some quantitative differences between halothane and propofol interactions with the two Ca^{2+}-ATPases. For halothane, the intrinsic ΔG is more negative for PMCA than for SERCA1. For propofol, the ΔG is more favorable as compared to halothane, and the values are similar for both enzymes (−5.2 *versus* −5.1 kcal/mol at 25 °C). No published data for propofol interaction with soluble proteins are available for comparison.

As described above, similar values were obtained for both enzyme preparations, one PMCA solubilized in detergent and the other SERCA1 studied in the SR membrane. This similarity is especially striking for propofol, a compound of high lipid solubility. It suggests that the observed anesthetic effects on enzyme activity are due mainly to their direct binding in the hydrophobic regions of the protein and not to their partitioning into membrane lipids. This notion is concordant with the results of [^{14}C]halothane photoaffinity labeling experiments. We have recently demonstrated that approximately comparable (40−50) percentage of saturable [^{14}C]halothane binding to SR membranes is localized to the SERCA1 protein and lipids (Kosk-Kosicka et al., 1997). In addition, anesthetic binding to discrete sites in the enzyme molecule is indicated by heterogeneous distribution of the label among Ca^{2+}-ATPase fragments produced by controlled tryptic digestion (Kosk-Kosicka et al., 1997).

While the free energy of halothane binding to the Ca^{2+}-ATPase sites is in the range reported for VA binding to soluble proteins, the source of the free energy change that drives the interactions is apparently different for the studied soluble *versus* membrane proteins. The entropy-driven binding of the anesthetics to the Ca^{2+}-ATPases contrasts with the enthalpy-driven binding of VA described for soluble proteins such as firefly luciferase, BSA, and myoglobin (Ueda & Kamaya, 1973; Dickinson et al., 1993; Ewig & Maestas, 1970). For example, $\Delta H = -4.32$ kcal/mol and $\Delta S = 0.66$ cal mol^{-1} K^{-1} were obtained for halothane binding to firefly luciferase at 20 °C (as recalculated from Dickinson et al. (1993)]. The uncovered difference in the contribution of the entropic and enthalpic components in the process of anesthetic interaction with membrane *versus* soluble proteins may reflect a greater role of nonpolar interactions in anesthetic binding to the ATPases, intrinsic membrane proteins, in contrast to significant electrostatic contributions in their binding to soluble proteins (Eckenhoff & Johansson, 1997). Also the temperature dependence of the anesthetic−protein interactions is in agreement with the involvement of hydrophobic bonding and is different (opposite direction of changes) from the temperature dependence reported for luciferase (Dickinson et al., 1993).

Contributions to the Positive Entropy Change. (a) Conformational Entropy. The entropy-driven binding of anesthetics to the ATPases could be compared to antagonist−receptor binding which in several reports (although not consistently in all studies) was revealed to contrast with the enthalpy-driven binding of agonists to the same receptor (Weiland et al., 1979, 1980; Contreras et al., 1986; Raffa & Porreca, 1989). By analogy, anesthetic (antagonist) binding to the ATPases might prevent the normal conformational transition which the enzymes undergo upon Ca^{2+} and ATP (agonists) binding to efficiently catalyze Ca^{2+} transport across the membrane (Inesi et al., 1980; Stokes & Lacapere, 1994). We have shown previously that inhibition of PMCA Ca^{2+}-ATPase activity by halothane and other VA correlates well with the elimination of the Ca^{2+}-induced conformational change (Lopez & Kosk-Kosicka, 1995).

(b) Desolvation of the Cavity and the Ligand. Another source of the observed positive entropy change is displacement of ordered water molecules from around the anesthetic and the nonpolar binding site(s) (pocket/cavity) in the Ca^{2+}-ATPase, if they are normally hydrated. Recent reports indicate that hydrophobic cavities of various proteins contain water molecules, even though they cannot be detected by crystallographic analysis and, thus, were previously believed to be empty (Ernst et al., 1995; Buckle et al., 1996; Varadarajan & Richards, 1992). The significantly bigger intrinsic entropy change observed for propofol (same for both enzymes: 33 cal mol^{-1} K^{-1}) than for halothane (28 cal mol^{-1} K^{-1} for PMCA *versus* 24 cal mol^{-1} K^{-1} for SERCA1) is in agreement with displacement of more water molecules. As the molecule of propofol is bigger and an order of magnitude more hydrophobic than halothane [respective values for octanol/water partitioning are 6200 and 200 (Hansch et al., 1995)], more water molecules could be displaced into bulk water from desolvation of the anesthetic and from its binding site in the protein. The fact that the interaction is least favorable for halothane in Me_2SO (ΔG is 0.9 kcal/mol less for PMCA and 0.5 kcal less for SERCA1 for halothane in Me_2SO than in water) and is most favorable for propofol which is dissolved in Me_2SO (ΔG is 2.6−2.7 kcal/mol more for propofol than for halothane in Me_2SO) suggests that the lower entropy contribution is characteristic of the anesthetic itself as opposed to the solvent effect.

(c) Immobilization of the Ligand in the Binding Site. The so-called cratic entropy estimates range from −8 to −85 cal mol^{-1} K^{-1}; there is little agreement on which is most correct for protein−ligand imteractions (Finkelstein & Janin, 1989; Kaufmann, 1959).

Possible Localization of the Anesthetic Binding Site(s). The calculated volumes for halothane and propofol are 132 and 247 Å^3, respectively, while the volume for bulk water molecule is 30 Å^3. Under close packing conditions (as in a cavity), a water molecule occupies 15.5 Å^3 when modeled as a 1.4 Å radius sphere (Varadarajan & Richards, 1992). Anesthetic molecules packed in the cavities may also occupy respectively smaller volumes. The cavities in PMCA and SERCA1 in which the anesthetics bind are expected to be of the same order of magnitude as the anesthetics. The volumes of internal cavities calculated from X-ray and NMR data for numerous proteins of molecular mass below 35 kDa range from 10^3 to 221 Å^3 (Eriksson et al., 1992; Rashin et al., 1986). Thus, for the Ca^{2+}-ATPases of molecular mass 110−136 kDa, binding of anesthetics in internal cavities is highly probable. Their localization could be either on the protein−protein interface, deep in the protein molecule, or on the protein−lipid interface. Anesthetic occupancy of any such cavities could alter protein dynamics and result in the described disturbance of the catalytic cycle of the enzyme (Lopez & Kosk-Kosicka, 1995). For example, the activation observed only for SERCA1 at low halothane concentrations

could result from anesthetic binding in the interfaces of monomers constituting enzyme oligomers, thus causing dissociation of the oligomers (Karon et al., 1994). Anesthetic binding in the protein interior could occur in the functionally well-defined Ca^{2+}-ATPase cavities, where calcium ions bind in the transmembrane channel or where ATP binds and phosphorylation of the enzyme occurs in the big cavity in the globular cytoplasmic portion of the enzyme. For example, the hydrophobic pocket in adenylate kinase in which halothane binding was revealed by X-ray analysis (Sachsenheimer et al., 1977) is the binding site for the substrate AMP, and there is high homology between the primary and secondary structure of nucleotide-binding sites and phosphorylation sites in Ca^{2+}-ATPases and kinases (MacLennan et al., 1985).

Based solely on the molecular volumes of the two anesthetics, we expect a 2:1 molar ratio of halothane to propofol binding to the Ca^{2+}-ATPases. Taking into account the differences in chemical properties of the two anesthetics (structure, size, hydrophobicity) and the observation that propofol has 2 orders of magnitude higher affinity for both enzymes, we consider their binding in different sites within the same cavity. One intriguing possibility is the large cavity containing the nucleotide-binding and phosphorylation sites. The first site also binds *p*-nitrophosphophenyl (used in kinetic studies as a pseudosubstrate) and thus it might also bind propofol. The second site which binds charged molecules such as phosphate (a substrate), orthovanadate, and possibly fluorides that have been recently shown to form intermediate state analogues might bind halothane (Murphy & Coll, 1992 a,b; Troullier et al., 1992).

Solvent Effect. For both enzymes, ΔG is most negative for propofol (in Me_2SO), followed by halothane in water, and then halothane in Me_2SO, indicating that Me_2SO does not facilitate anesthetic binding. In fact, in the presence of any of the three solutes, significantly higher halothane concentrations are required for half-maximal inhibition than in the "pure" aqueous solution. The comparison (Figures 2 and 3A) explains the apparent discrepancy in the values reported in literature. For example, the k_{Hal} for the VA delivered from saturated aqueous solution, which is generally accepted as the most appropriate method for VA delivery in *in vitro* experiments (Diamond & Berman, 1980; Blanck, 1981; Dubois & Evers, 1992; Dickinson et al., 1993), is respectively 1.8- and 2.6-fold lower for the SR enzyme than in the presence of DMF [used by Karon and Thomas (1993)] and Me_2SO, respectively. The solute effect is not easy to interpret. At low concentrations corresponding to maximally 0.0074 mole fraction of Me_2SO (Dupont & Pougeois, 1983) used in our experiments (2% Me_2SO), none of the three solutes (in the absence of halothane) has any significant effect on Ca^{2+}-ATPase activity (Kosk-Kosicka et al., 1994; Kosk-Kosicka & Roszczynska, 1994). Stabilizing effects reported for various proteins, including protection against loss of activity upon cold storage or mechanical denaturation, were reported at distinctly higher concentrations (20−40%) of DMF or Me_2SO (Asakura et al., 1978; Ma & Wang, 1983; George et al., 1969; Fontes et al., 1995). The effects that Me_2SO has on proteins are believed to be by indirect water-mediated interactions through exclusion or replacement of water molecules (Collins & Washabaugh, 1985; Singer, 1962). One possible scenario is that solute molecules bind to halothane and thus protect it from interaction with the sites in the protein. Alternatively, they may affect the cavity where the anesthetic binds such that halothane has less accessibility to the enzyme. The results cannot be explained by solvent polarity since the most polar (after water) Me_2SO has the biggest "protective" effect against halothane inhibition of both enzymes. While at present not enough supporting information is available to explain the observed solute effects, the fact that they are comparable for both enzymes indicates a mechanism applicable to both.

Summary. The thermodynamic analysis supports anesthetics' binding to both Ca^{2+}-ATPases in an entropy-driven process. Application of two models, using independent calculations (that respectively provide intrinsic and actual thermodynamic parameters), allows us to conclude the importance of entropic contributions. We have discussed the contributions to the total entropy change (conformational, dehydration of the anesthetic and binding site, immobilization of the ligand in the binding site). All contributions are expected to be bigger for propofol (a bigger, nonpolar molecule) than halothane. The cumulative effect is that the entropy change overcompensates the enthalpy change and drives the interaction. Organic solutes, such as Me_2SO, apparently exert some protective effects against anesthetic inhibition of the enzymes.

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