Spectroscopic Analysis of Halothane Binding to the Plasma Membrane Ca^{2+}-ATPase

Maria M. Lopez and Danuta Kosk-Kosicka
Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, and Department of Anesthesiology/Critical Care Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287 USA

ABSTRACT The intrinsic tryptophan (Trp) fluorescence of the plasma membrane Ca^{2+}-ATPase (PMCA) is significantly quenched by halothane, a volatile anesthetic common in clinical practice. It has been proposed that halothane inhibition of the Ca^{2+}-ATPase activity results from conformational changes following anesthetic binding in the enzyme. We have investigated whether the observed quenching reflects halothane binding to PMCA. We have shown that the quenching is dose dependent and saturable and can be fitted to a binding curve with an equilibrium constant $K_{Hal} = 2.1$ mM, a concentration at which the anesthetic approximately half-maximally inhibits the Ca^{2+}-ATPase activity. The relatively low sensitivity of halothane quenching of Trp fluorescence to the concentration of phosphatidylcholine and detergent in the PMCA preparation concurs with the quenching resulting from anesthetic binding in the PMCA molecule. Analysis of the Trp fluorescence quenching by acrylamide indicates that the Trp residues are not considerably exposed to the solvent (Stern-Volmer quenching constant of 2.9 M^{-1}) and do not differ significantly in their accessibility to halothane. Other volatile anesthetics, diethyl ether and diisopropyl ether, reduce the quenching caused by halothane in a dose-dependent manner, suggesting halothane displacement from its binding site(s). These observations indicate that halothane quenching of intrinsic Trp fluorescence of PMCA results from anesthetic binding to the protein. The analysis, used as a complementary approach, provides new information to the still rudimentary understanding of the process of anesthetic interaction with membrane proteins.

INTRODUCTION

The molecular site and mechanism of action of volatile anesthetics (VAs) remain controversial. Two major hypotheses have been developed that consider lipids or proteins as molecular targets of action (for a review, see Miller, 1985; Koblin, 1990; and Franks and Lieb, 1994). In recent years membrane proteins have been the focus of attention. We have previously identified an intrinsic membrane protein, the plasma membrane Ca^{2+}-ATPase (PMCA), as a potential target for anesthetic action (Kosk-Kosicka and Roszczynska, 1993; Kosk-Kosicka, 1994). We have suggested that volatile anesthetics bind in hydrophobic cavities in the protein, as has been demonstrated for small ligands such as Xenon, whose binding to metmyoglobin affects the protein conformation (Schoenborn and Featherstone, 1967; Tilton et al., 1984). In PMCA, the Ca^{2+}-ATPase activity, the Ca^{2+}-dependent conformational change that the protein undergoes upon Ca^{2+} binding in the initial step of the enzymatic cycle, and the intrinsic Trp fluorescence (11 Trp residues) are affected by volatile anesthetics in a dose-dependent manner (Lopez and Kosk-Kosicka, 1995). We have also shown for the first time that the effects of halothane on the Ca^{2+}-ATPase of the intracellular sarcoplasmic reticulum (SERCA1) are the result of direct binding of the anesthetic to that protein (Kosk-Kosicka et al., 1997). In the present study we are investigating the properties and the environment of the halothane-binding sites in the PMCA by fluorescence spectroscopy. The quenching of the protein intrinsic tryptophan fluorescence by volatile anesthetics has been successfully used by Johansson et al. (1995) to measure halothane binding to bovine serum albumin. The results obtained with this approach are comparable to the ones obtained using photoaffinity labeling (Eckenhoff and Shuman, 1993) and fluorine 19 NMR spectroscopy coupled with gas chromatography partition analysis (Dubois et al., 1993). The good correspondence between different methodologies allows us to study halothane binding to PMCA by using the protein Trp fluorescence (none of the other techniques can be used for this protein partly because of its low concentration even after purification). Our results indicate that there is a direct interaction of the volatile anesthetic with the plasma membrane Ca^{2+}-ATPase. The anesthetic hydrophobic binding site(s) is not exposed to the solvent, and halothane is displaced from the cavity(ies) in the protein by other volatile anesthetics (ethers). The conformational changes in the PMCA due to the interaction of the protein with the anesthetic are not as dramatic as the changes produced in the enzyme by urea, a protein denaturant.

Received for publication 10 July 1997 and in final form 28 October 1997.

Address reprint requests to Dr. Maria M. Lopez, Department of Chemistry and Biochemistry, Texas Tech University, Mail Stop 1061, Lubbock, TX 79409-1061. Tel.: 806-742-3062; Fax: 806-742-1289; E-mail: qemar@ttacs.ttu.edu.

0006-3495/98/02/974/07 $2.00

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 obtained from Nikko (Tokyo, Japan). Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia LKB Biotechnology instructions as described earlier (Kosk-Kosicka and

Bzdega, 1988). Thymol-free halothane was obtained from Halocarbon Laboratories (River Edge, NJ).

The methods used for PMCA purification and protein determination were as described previously (Kosk-Kosicka et al., 1986; Kosk-Kosicka and Bzdega, 1988). Briefly, the enzyme was purified from erythrocyte membrane ghosts by calmodulin affinity column chromatography in the presence of the nonionic detergent $C_{12}E_8$, which led to preparations of >95% pure protein, as judged by silver staining electrophoreses. Protein concentration was measured by Bio-Rad protein microassay. Sarcoplasmic reticulum was prepared from rabbit skeletal muscle in the laboratory of Dr. Inesi as described previously (Eletr and Inesi, 1972; Kosk-Kosicka et al., 1983).

Fluorescence measurements

The tryptophan fluorescence intensity of the Ca^{2+}-ATPase was measured at equilibrium, with a Fluoromax spectrofluorometer with DM3000F software. Tryptophan fluorescence was excited at 290 nm, and the emission was recorded at 330 nm. The reaction mixture contained 100 mM Tris-HCl (pH 7.4), 0.15 mM $C_{12}E_8$, 120 mM KCl, 8 mM $MgCl_2$, and 1 mM EGTA. Free Ca^{2+} was 17.5 μM, and the protein concentration was 50–70 nM (7–10 μg/ml). In experiments with sarcoplasmic reticulum (SR), $C_{12}E_8$ was omitted and the protein concentration was 10 μg/ml. The measurements were performed at 25°C in a total volume of 1.1 ml. The aliquots of halothane delivered to the assay were taken from solution of saturated volatile anesthetic in the reaction mixture, which was prepared daily from the stock of pure halothane under nitrogen gas at room temperature. Halothane and ethers were delivered to the cuvette in an airtight Hamilton syringe.

Determination of halothane concentration

Parallel to the fluorescence assay, samples were incubated under identical conditions and used to determine the effective anesthetic concentration by gas chromatography (Lopez and Kosk-Kosicka, 1997).

RESULTS

We have determined that several halogenated volatile anesthetics (halothane, metoxyflurane, isoflurane, and enflurane) quench the intrinsic tryptophan fluorescence of the plasma membrane Ca^{2+}-ATPase (Lopez and Kosk-Kosicka, 1995). The present study focuses on the effect of halothane on PMCA fluorescence intensity, using a wider halothane concentration range.

As shown in Fig. 1 *A* (*curve A*), the enzyme Trp fluorescence decreases in a dose-dependent manner, reaching saturation at 6–7 mM halothane. Considering independent and noninteractive binding sites, the fluorescence intensity varies according to the following equation:

$$I = \frac{Q_{max} \times [\text{halothane}]}{k_{Hal} + [\text{halothane}]} \quad (1)$$

where I is the fluorescence intensity at any halothane concentration, Q_{max} is the maximum fluorescence that can be quenched, and k_{Hal} is the equilibrium (dissociation) constant for the anesthetic-protein interaction. The data points were fitted using the nonlinear square regression method, and the values obtained for Q_{max} and k_{Hal} were 1.08 ± 0.02 and 2.1 ± 0.1 mM, respectively. Several controls were considered. Fig. 1 *A* (*curve B*) shows the effect of halothane (up to 9 mM) on free L-tryptophan (10 μM). A linear decrease of the free Trp fluorescence was observed, with a maximum quenching of 17 ± 1% at 9 mM halothane. The bromine atom in the halothane molecule is the most likely cause of the quenching (although the chlorine atom may also contribute). We measured the effect of sodium bromide on

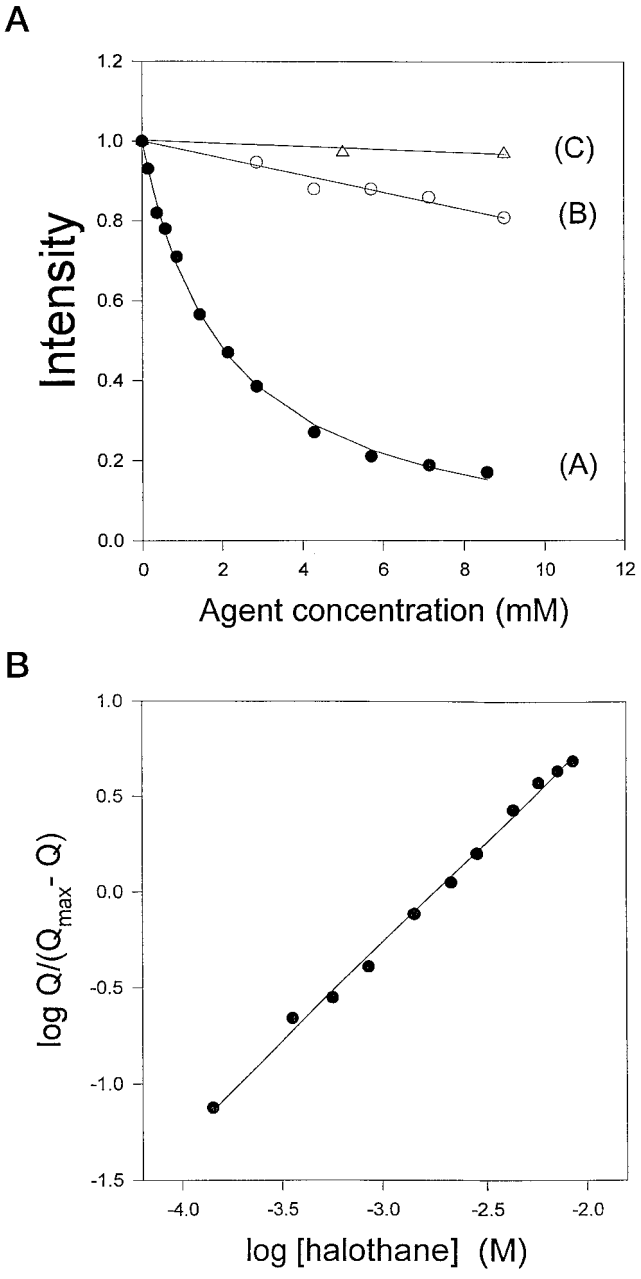

FIGURE 1 (A) Effect of halothane (*A*) and NaBr (*C*) on PMCA Trp fluorescence (B). Effect of halothane on free L-tryptophan fluorescence. The reaction mixture contained 50 mM Tris-maleate (pH 7.4), 120 mM KCl, 8 mM $MgCl_2$, 17.5 μM free Ca^{2+}, 150 μM $C_{12}E_8$, and 1 mM EGTA. Protein and free L-tryptophan concentrations were 50 nM and 10 μM, respectively. The experiments were performed at 25°C. (B) Hill plot for the halothane-PMCA interaction from curve A. The slope (Hill coefficient) is 1.04 + 0.02. Q is the quenched fluorescence; Q_{max} is the maximum fluorescence that can be quenched, as obtained from fitting curve A to Eq. 1 (under Results).

Ca^{2+}-ATPase (50 nM) fluorescence (Fig. 1 *A*, *curve C*) and found that 10 mM bromide ion causes a 3 ± 1% decrease in the enzyme Trp fluorescence. The low quenching by bromide ions supports the idea that the Trp(s) in the plasma membrane Ca^{2+}-ATPase was not exposed to the solvent. The slight effect of the bromide ions on the enzyme fluorescence also indicates that the free halothane in solution has a minor effect on the observed quenching of the Ca^{2+}-ATPase fluorescence.

A Hill plot of halothane binding to PMCA is shown in the Fig. 1 *B*. The Hill coefficient calculated by this approach is $n = 1.04 \pm 0.02$, indicating that there is no cooperativity in the halothane-PMCA interaction.

Acrylamide is known as an excellent uncharged quenching probe that is very sensitive to the exposure of tryptophans in proteins (Eftink and Ghiron, 1976). Fig. 2 shows the effect of acrylamide on the PMCA Trp fluorescence. Experimental points were fitted to Eq. 2:

$$I_o/I_i = 1 + K_{SV} \times [\text{acrylamide}] \quad (2)$$

where I_o and I_i are the intensities in the absence and in the presence of acrylamide, respectively, and K_{SV} is the Stern-Volmer quenching constant. A linear correlation is observed between the intensities and the acrylamide concentration, which suggests that all Trp(s) in the protein differ slightly in accessibility (Eftink and Ghiron, 1976). K_{SV}, obtained from the slope, is 2.9 ± 0.1 M^{-1}. The collisional quenching constant, obtained as $k_q = K_{SV}/\tau$, where τ is the lifetime of the Trp in the absence of acrylamide, is $5.9 \pm 0.3 \times 10^8$ $M^{-1}\ s^{-1}$ ($\tau = 4.9$ ns, from Lopez and Kosk-Kosicka, 1995).

We compared the effect of acrylamide on PMCA and SERCA1 (Fig. 2) for several reasons. SERCA1 and PMCA are similar enzymes, and the former has been studied extensively by numerous laboratories. Based on hydrophobicity plots, immunological localization of epitopes, chemical derivatization, and fluorescence spectroscopy measurements, substantial progress has been made in predicting its three-dimensional structure (for a review, see Inesi et al., 1990; Stokes et al., 1994; and Andersen and Vilsen, 1995). It appears that in SERCA1, 12 of 13 Trp are located near the membrane interface. Thus the comparison of the acrylamide effect on PMCA and SERCA1 may provide useful information about the PMCA Trp(s) environment.

We also observe a linear correlation between the intensities and the acrylamide concentrations for SERCA1, and the K_{SV} of 1.9 ± 0.5 M^{-1} is very comparable to the value obtained by Gryczynski et al. (1989) ($K_{SV} = 1.6\ M^{-1}$).

The small Stern-Volmer constant, meaning low accessibility to the quencher (Eftink and Ghiron, 1976), and the fact that all Trp(s) are equally accessible to the quencher support once more the theory that the Trp(s) in the PMCA and SERCA1 are not exposed to the solvent.

To assess if the effect of volatile anesthetics on their targets is similar to the effect of denaturants, we have included in our study the effect of the protein denaturant urea on the PMCA Trp fluorescence. Fig. 3 shows that urea also quenches the enzyme intrinsic fluorescence in a dose-dependent manner (2, 4, and 8 M urea quenched PMCA Trp fluorescence intensity by 8, 18, and 24 ± 2%, respectively). Another effect observed in the presence of certain urea concentrations is a shift in the maximum of the emission spectra: 8 M urea produces a 5–6-nm red shift (Fig. 3, *curve*

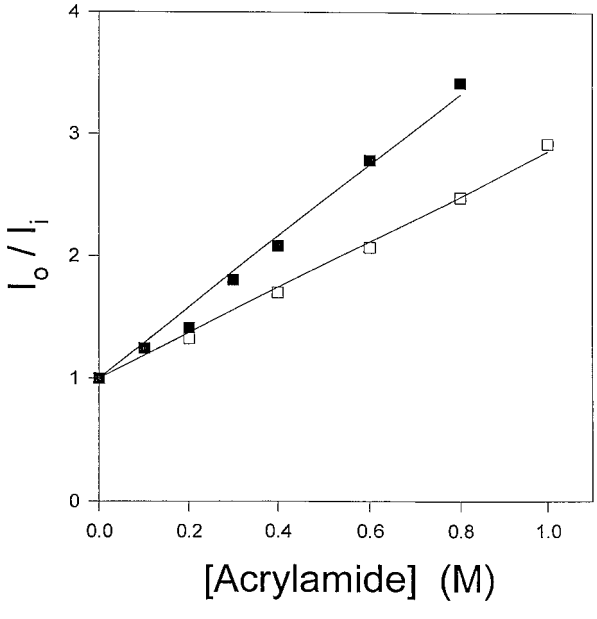

FIGURE 2 Effect of acrylamide on the PMCA (■) and SERCA1 (□) intrinsic tryptophan fluorescence. The protein concentration in both cases was 10 μg/ml. Data points were fitted to Eq. 2 (under Results). Experimental conditions were as described in Fig. 1.

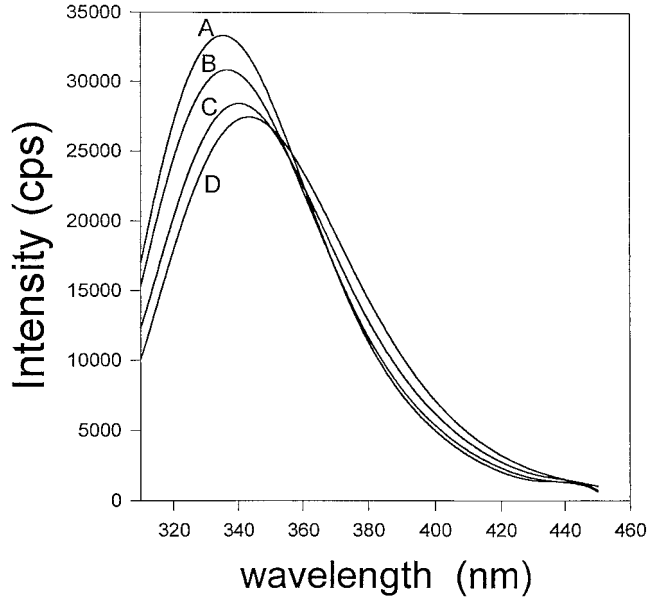

FIGURE 3 Fluorescence emission spectra of PMCA in the absence (*A*) and presence of 2 M (*B*), 4 M (*C*), and 8 M (*D*) urea. The excitation wavelength was 290 nm. Protein concentration was 65 nM. Experimental conditions were as described in Fig. 1.

D), indicating that under these experimental conditions the environment of the Trp(s) becomes more polar, as expected upon unfolding of the protein.

We then studied the possible influence of different phosphatidylcholine (PC) and $C_{12}E_8$ concentrations on the Ca^{2+}-ATPase fluorescence quenching by halothane. Table 1 shows that the enzyme Trp fluorescence quenching in samples without PC or in samples with 40 μM PC (our standard experimental conditions) differs by only 5%, whereas in samples with 200 μM PC, the difference, with respect to our standard conditions, is ~10%. These results suggest that the volatile anesthetic interacts mainly with the protein. Table 2 shows that the extent of PMCA Trp fluorescence quenched by 4.3 mM halothane decreases slightly when the $C_{12}E_8$ and PC concentrations are two- and fourfold higher than in our standard experimental conditions (150 μM $C_{12}E_8$ and 40 μM PC). From these results we conclude that the effects of detergent and lipids are very comparable and do not contribute significantly to the observed effect of halothane on the PMCA Trp fluorescence.

The nonpolar sites in proteins are limited in size and shape. Thus we have studied the effects of diethyl and diisopropyl ether (which by themselves do not affect the PMCA intrinsic Trp fluorescence) on the enzyme fluorescence quenching by halothane. At saturated halothane concentrations (7.1 mM), increasing the diethyl and diisopropyl ether concentrations decreases the halothane quenching (Fig. 4). These results suggest that halothane could be displaced from its binding site(s) in the protein. We also observe that the effect of diisopropyl ether is larger than the effect of diethyl ether: in the presence of 9% (v/v) diisopropyl or diethyl ether, the halothane quenching observed is 42% and 61%, respectively (versus 80% in the absence of any ether) (Fig. 4). Thus different ethers may have different competitive binding constants when interacting with PMCA in the presence of halothane.

DISCUSSION

We have shown that halothane quenches the PMCA Trp fluorescence in a saturable manner. We interpret the quenching effect to be a result of a direct interaction (although weak, K_{Hal} in mM range) between halothane and the PMCA based on the following findings: 1) 7 mM halothane quenches the PMCA Trp fluorescence by 80%, whereas it quenches the free Trp fluorescence by only 10–12%; 2) the halothane quenching of the protein fluorescence can be fitted to a binding curve, whereas the halothane quenching of the free Trp fluorescence follows a linear correlation, characteristic of nonspecific binding; 3) there is a strong correlation between the halothane concentration that half-maximally inhibits the Ca^{2+}-ATPase activity (I_{50} = 2.6 mM; Lopez and Kosk-Kosicka, 1995) and the equilibrium constant obtained from the quenching experiments (K_{Hal} = 2.1 mM). Similar spectroscopic results have been obtained for the interaction of halothane with bovine serum albumin, whereas no significant fluorescence quenching has been observed for another protein (apomyoglobin) (Johansson et al., 1995). Other proteins have been shown, by photoaffinity labeling, to saturably bind halothane: the Ca^{2+}-ATPase in the sarcoplasmic reticulum (Kosk-Kosicka et al., 1997) and rat brain synaptosomes (El-Maghrabi et al., 1992).

Several observations have led us to conclude that the PMCA Trp(s) are not exposed to the solvent: 1) bromide ions do not quench the PMCA Trp fluorescence significantly (water-soluble heavy atoms are known to have little access to buried tryptophanyl residues; Bogomolni et al.,

TABLE 1 Effect of phosphatidylcholine on halothane quenching of PMCA tryptophan fluorescence

[Halothane] (mM)	Quenching (%)		
	No PC	40 μM PC	200 μM PC
1.43	42	43	40
2.85	58	62	56
4.28	68	72	65
5.71	75	79	ND
7.14	80	81	ND

ND, Not done.

TABLE 2 Effect of $C_{12}E_8$ and phosphatidylcholine on halothane quenching of the tryptophan fluorescence in the PMCA

[$C_{12}E_8$] (μM)	[PC] (μM)	Quenching (%)
150	40	72
300	40	64
150	150	63

The halothane concentration was 4.3 mM.

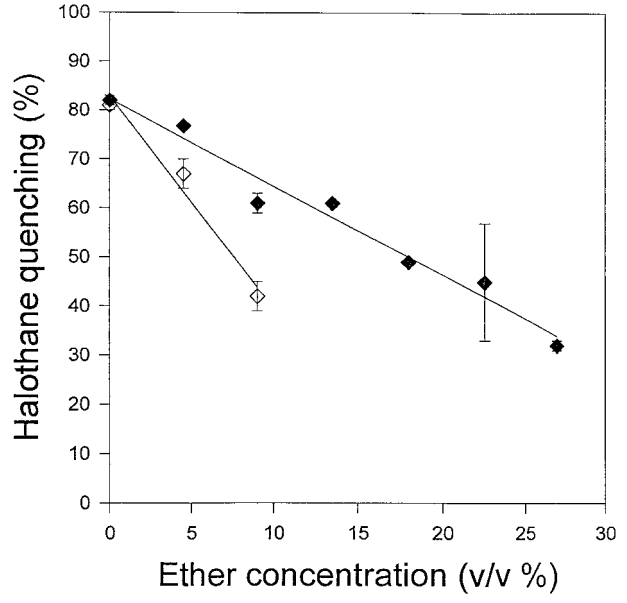

FIGURE 4 Effect of diethyl ether (♦) and diisopropyl ether (◇) on halothane quenching of PMCA Trp fluorescence. Halothane concentration was 7.1 mM. Experimental conditions were as described in Fig. 1. The line through the data points was generated by linear regression analysis.

1978); 2) the PMCA fluorescence emission spectrum has a maximum at 330 nm (Lopez and Kosk-Kosicka, 1995), which is characteristic of Trp(s) residues buried in the protein (Burstein et al., 1973; Lakowicz, 1991); 3) the dynamic quenching experiments with acrylamide show a linear Stern-Volmer plot, suggesting that all Trp differ slightly in accessibility (Eftink and Ghiron, 1976). The Stern-Volmer quenching constants for the acrylamide interaction with PMCA and SERCA1 are similar (2.9 and 1.9 M^{-1}, respectively). The low K_{SV} value obtained for SERCA1 agrees with the fact that most of its Trp(s) are in the membrane. Both enzymes have Stern-Volmer quenching constants that are comparable to other multitryptophan proteins (for trypsin $K_{SV} = 2.4\ M^{-1}$, trypsinogen $K_{SV} = 2.3\ M^{-1}$; Eftink and Ghiron, 1976). For proteins in which the Trp(s) are fairly exposed to the quencher, higher Stern-Volmer quenching constants are obtained (i.e., pepsin $K_{SV} = 9.5\ M^{-1}$, papain $K_{SV} = 8.0\ M^{-1}$; Eftink and Ghiron, 1976).

In conclusion, it appears that most of the tryptophans in the plasma membrane Ca^{2+}-ATPase are not considerably exposed to the solvent. This result is in agreement with the expected membrane organization of the enzyme (based on the model for SR Ca^{2+}-ATPase; MacLennan et al., 1985) and with the enzyme predicted secondary structure (based on the alignment of transport ATPases; Carafoli and Chiesi, 1992; Carafoli, 1994).

Comparison of the quenching effects of volatile anesthetics and the denaturant urea

We have shown that the effect of VA on the plasma membrane Ca^{2+}-ATPase bears some resemblance to the effect of urea on this enzyme. The fact that similar effects have been observed for other proteins led to the suggestion that anesthetics might behave as denaturants (Mitaku et al., 1995). Among the similarities, we found that volatile anesthetics and urea quench the PMCA Trp fluorescence, produce a red shift in the maximum of the emission spectra, and inhibit the Ca^{2+}-ATPase activity (Lopez and Kosk-Kosicka, 1995; Kosk-Kosicka et al., 1994; Fig. 3). However, volatile anesthetics and the denaturant urea differ in the extent of quenching, the conformational changes they produce, and the correlation between quenching and loss of the PMCA activity. We have observed that 8 M urea (under these conditions most proteins are considered unfolded) quenches the PMCA Trp fluorescence by 24%, and the maximum of the emission spectrum is red shifted by ~5 nm. However, the enzyme activity is already completely inhibited at 2 M urea (Kosk-Kosicka et al., 1994), which quenches the protein Trp fluorescence by only 6–8% and produces no red shift in the maximum of the emission spectrum (Fig. 3). So the Ca^{2+}-ATPase activity is affected at a much lower urea concentration than that required to unfold the protein. Such differences in concentration required for inhibition of the enzyme activity and protein unfolding have also been reported for other enzymes (Zhou et al., 1993; West et al., 1990). In contrast to describing anesthetic action on proteins as denaturating, we have proposed that binding of volatile anesthetics perturbs conformational substate(s) of the enzyme, leading to the observed inhibition of the Ca^{2+}-ATPase activity (Lopez and Kosk-Kosicka, 1995). The good correlation between the changes in the conformation of the protein (followed by fluorescence spectroscopy) and the loss of enzyme activity supports this idea. Another important difference between denaturants and volatile anesthetics is that the unfolding process for PMCA is irreversible (as it is for most membrane proteins; for a review see Haltia and Freire, 1995; for SERCA1 see Lepock et al., 1990 and Merino et al., 1994): the enzyme activity is not recovered after heat denaturation. The effect of volatile anesthetics is reversible: the Ca^{2+}-ATPase activity is recovered after evaporation of the anesthetic (Kosk-Kosicka and Roszczynska, 1993).

Thus the anesthetics' effects on PMCA may not be similar to the effect of denaturants: 1) the conformational changes in the protein due to the anesthetic binding are not drastic and are well correlated with the impairment of the enzyme activity, as opposed to the denaturant effects; and 2) the anesthetic-protein interaction is a reversible process, whereas the unfolding process of the protein is not.

Effect of lipids and detergent on the PMCA fluorescence quenching by halothane

The long-accepted theory that the effect of volatile anesthetics is through lipids has been changing. We have shown that different PC concentrations seem to affect the halothane quenching very little (the increase of PC concentration by fivefold with respect to our standard experimental conditions (200 μM and 40 μM, respectively) decreases halothane quenching by only 10–12%; Table 1). These results agree with our hypothesis that the anesthetics interact directly with the protein. We have been able to prove this for the Ca^{2+}-ATPase in the sarcoplasmic reticulum by using photoaffinity labeling: up to 50% of [^{14}C]halothane is incorporated into the enzyme (Kosk-Kosicka et al., 1997).

On the other hand, we have to consider that the PMCA used in this study is a purified enzyme that contains detergent. We have shown that the effect of $C_{12}E_8$ on the halothane quenching is very comparable to the PC effect: in the range of concentrations studied, either detergent or lipids decrease the halothane quenching by a maximum of 10–12%. These results suggest that under our experimental conditions, the quencher does not partition overwhelmingly into the lipid or micellar phase, or if it does, the excess anesthetic left is enough to quench the protein fluorescence and is not dramatically dependent on the PC or $C_{12}E_8$ concentrations. Different observations have been published for the interaction of bromostearate with spectrin (Kahana et al., 1992): quenching of spectrin fluorescence by bromostearate is totally suppressed by low concentrations of nonionic

detergent and by the presence of vesicles. The authors conclude that the ligand partitions entirely into the micellar phase or vesicles, respectively.

Reversibility of PMCA-halothane interaction by ethers

We have shown that two nonquenching anesthetics, diethyl and diisopropyl ether, can reverse in part the halothane quenching effect on PMCA fluorescence. At saturated halothane concentration (7.1 mM), 22–23% (v/v) of diethyl ether is needed to inhibit the halothane quenching by 50% (Fig. 4). The observed apparent competition between halothane and diethyl ether may indicate that the two anesthetics bind in the same hydrophobic cavity(ies) in the protein, or that diethyl ether binds in a different place but affects halothane binding, significantly decreasing PMCA affinity for halothane. Halothane displacement from its binding site by diethyl ether has also been shown for a globular protein (BSA) (Johansson et al., 1995). On the other hand, Raines and McClure did not observe any significant effect of diethyl ether on the halothane quenching of the nicotinic acetylcholine receptor's (nAcChoR's) Trp fluorescence (Raines and McClure, 1997). This result may be just a consequence of their experimental design: 1 mM halothane quenches nAcChoR's Trp fluorescence by ~10%, so if diethyl ether binds in the same place as halothane, but with a lower affinity, the diethyl ether binding to the protein in the presence of 1 mM halothane would not affect the halothane quenching. On the contrary, if the protein is saturated with halothane, it is easier to observe competition between the two volatile anesthetics.

We have also observed that another ether (diisopropyl ether) competes with halothane, but has a much larger effect on halothane quenching than diethyl ether (Fig. 4). This result may indicate that PMCA affinity for diisopropyl ether is higher than the affinity for diethyl ether. We have already shown that PMCA binds another general anesthetic (propofol) with an affinity that is one to two orders of magnitude higher than that of halothane (Lopez and Kosk-Kosicka, 1997). Propofol is a larger molecule than halothane (ratio of volumes ~1.8), and the interaction of the former with the protein is more favorable thermodynamically because of a higher entropy change upon propofol binding (Lopez and Kosk-Kosicka, 1997). As we have shown that the anesthetic–Ca^{2+}-ATPase interaction is an entropy-driven process, most probably the entropy change that occurs upon diisopropyl ether binding to the enzyme is greater than for diethyl ether (the ratio of the volumes for the ether molecules is ~1.4), which would explain the difference in the PMCA affinity for the two ethers.

This work was supported by grant GM 447130 from the National Institutes of Health and grant 92010190 from the American Heart Association.

REFERENCES

Andersen, J. P., and B. Vilsen. 1995. Structure-function relationships of cation translocation by Ca^{2+} and Na^+,K^+-ATPases studied by site-directed mutagenesis. *FEBS Lett.* 359:101–106.

Bogomolni, R. A., L. Stubbs, and J. K. Lanyi. 1978. Illumination-dependent changes in the intrinsic fluorescence of bacteriorhodopsin. *Biochemistry.* 17:1037–1041.

Burstein, E. A., N. S. Vedenkina, and M. N. Ivkova. 1973. Fluorescence and the localization of tryptophan residues in protein molecules. *Photochem. Photobiol.* 18:263–279.

Carafoli, E. 1994. Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB J.* 8:993–1002.

Carafoli, E., and M. Chiesi. 1992. Calcium pumps in the plasma and intracellular membranes. *Curr. Top. Cell. Regul.* 32:209–241.

Dubois, B. W., S. F. Cherian, and A. S. Evers. 1993. Volatile anesthetics compete for common binding sites on bovine serum albumin: a ^{19}F-NMR study. *Proc. Natl. Acad. Sci. USA.* 90:6478–6482.

Eckenhoff, R. G., and H. Shuman. 1993. Halothane binding to soluble proteins determined by photoaffinity labeling. *Anesthesiology.* 79: 96–106.

Eftink, M. R., and C. A. Ghiron. 1976. Exposure of tryptophanyl residues in proteins. Quantitative determination by fluorescence quenching studies. *Biochemistry.* 15:672–679.

Eletr, S., and G. Inesi. 1972. Phase changes in the lipid moieties of sarcoplasmic reticulum membranes induced by temperature and protein conformational changes. *Biochim. Biophys. Acta.* 290:178–185.

El-Maghrabi, E. A., R. G. Eckenhoff, and H. Shuman. 1992. Saturable binding of halothane to rat brain synaptosomes. *Proc. Natl. Acad. Sci. USA.* 89:4329–4332.

Franks, N. P., and W. R. Lieb. 1994. Molecular and cellular mechanisms of general anesthesia. *Nature.* 367:607–614.

Gryczynski, I., W. Wiczk, G. Inesi, T. Squier, and J. R. Lakowicz. 1989. Characterization of the tryptophan fluorescence from sarcoplasmic reticulum adenosinetriphosphstase by frequency-domain fluorescence spectroscopy. *Biochemistry.* 28:3490–3498.

Haltia, T., and E. Freire. 1995. Forces and factors that contribute to the structural stability of membrane proteins. *Biochim. Biophys. Acta.* 1228: 1–27.

Inesi, G., C. Sumbilla, and M. E. Kirtley. 1990. Relationships of molecular structure and function in Ca^{2+}-transport ATPase. *Physiol. Rev.* 70: 749–760.

Johansson, J. S., R. G. Eckenhoff, and P. L. Dutton. 1995. Binding of halothane to serum albumin demonstrated using tryptophan fluorescence. *Anesthesiology.* 83:316–324.

Kahana, E., J. C. Pinder, K. S. Smith, and W. B. Gratzer. 1992. Fluorescence quenching of spectrin and other red cell membrane cytoskeletal proteins. Relation to hydrophobic binding sites. *Biochem. J.* 282:75–80.

Koblin, D. D. 1990. Mechanisms of action. *In* Anesthesia, Vol. 1. R. D. Miller, editor. Churchill Livingstone, New York. 51–83.

Kosk-Kosicka, D. 1994. Plasma membrane Ca^{2+}-ATPase as a target for volatile anesthetics. *Adv. Pharmacol.* 31:313–322.

Kosk-Kosicka, D., and T. Bzdega. 1988. Activation of the erythrocyte Ca^{2+}-ATPase either by self-association or interaction with calmodulin. *J. Biol. Chem.* 263:18184–18189.

Kosk-Kosicka, D., I. Fomitcheva, M. M. Lopez, and R. G. Eckenhoff. 1997. Heterogeneous halothane binding in the SR Ca^{2+}-ATPase. *FEBS Lett.* 402:189–192.

Kosk-Kosicka, D., M. Kurzmack, and G. Inesi. 1983. Kinetic characterization of detergent solubilized sarcoplasmic reticulum adenosinetriphosphatase. *Biochemistry.* 22:2559–2567.

Kosk-Kosicka, D., and G. Roszczynska. 1993. Inhibition of the plasma membrane Ca^{2+}-ATPase activity by volatile anesthetics. *Anesthesiology.* 79:774–780.

Kosk-Kosicka, D., S. Scaillet, and G. Inesi. 1986. The partial reactions in the catalytic cycle of the calcium-dependent adenosine triphosphatase purified from erythrocyte membranes. *J. Biol. Chem.* 261:3333–3338.

Kosk-Kosicka, D., A. Wawrzynow, and G. Roszczynska. 1994. Stabilizing and destabilizing effects on plasma membrane Ca^{2+}-ATPase activity. *Mol. Cell. Biochem.* 139:1–9.

Lakowicz, J. R. 1991. Topics in Fluorescence, Vol. 2. J. R. Lakowicz, editor. Plenum Press, New York.

Lepock, J. R., A. M. Rodahl, C. Zhang, M. L. Heynen, B. Waters, and K-H. Cheng. 1990. Thermal denaturation of the Ca^{2+}-ATPase of sarcoplasmic reticulum reveals two thermodynamically independent domains. *Biochemistry.* 29:681–689.

Lopez, M. M., and D. Kosk-Kosicka. 1995. How do volatile anesthetics inhibit Ca^{2+}-ATPases? *J. Biol. Chem.* 270:28239–28245.

Lopez, M. M., and D. Kosk-Kosicka. 1997. Entropy driven interactions between anesthetics and membrane proteins. *Biochemistry.* 36: 8864–8872.

MacLennan, D. H., C. J. Brandly, B. Korczak, and N. M. Green. 1985. Amino-acid sequence of a Ca^{2+}-Mg^{2+}-dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature.* 316:696–700.

Merino, J. M., J. V. Moller, and C. Gutierrez-Merino. 1994. Thermal unfolding of monomeric Ca(II), Mg(II)-ATPase from sarcoplasmic reticulum of rabbit skeletal muscle. *FEBS Lett.* 343:155–159.

Miller, K. W. 1985. The nature of the site of general anesthesia. *Int. Rev. Neurobiol.* 27:1–61.

Mitaku, S., S. Keiko, S. Odashima, K. Ikuta, M. S. Suwa, F. Kukita, M. Ishikawa, and H. Itoh. 1995. Interaction stabilizing tertiary structure of bacteriorhodopsin studied by denaturation experiments. *Proteins.* 22: 350–362.

Raines, D. E., and K. B. McClure. 1997. Halothane interactions with nicotinic acetylcholine receptor membranes. *Anesthesiology.* 86: 476–486.

Schoenborn, B. P., and R. M. Featherstone. 1967. Molecular forces in anesthesia. *Adv. Pharmacol.* 5:1–17.

Stokes, D. L., W. R. Taylor, and N. M. Green. 1994. Structure, transmembrane topology and helix packing of P-type ion pumps. *FEBS Lett.* 346:32–38.

Sumbilla, C., T. Cantalina, J. H. Collins, H. Malak, J. R. Lakowicz, and G. Inesi. 1991. Structural perturbation of the transmembrane region interferes with calcium binding in the Ca^{2+} transport ATPase. *J. Biol. Chem.* 266:12682–12699.

Tilton, R. F., I. D. Kuntz, and G. A. Petako. 1984. Cavities in proteins: structure of a metmyoglobin-xenon complex solved to 1.9 Å. *Biochemistry.* 23:2849–2857.

West, S. M., S. M. Kelly, and N. C. Price. 1990. The unfolding and attempted refolding of cytrate synthase from pig heart. *Biochim. Biophys. Acta.* 1037:332–336.

Zhou, H. M., X. H. Zhang, and C. L. Tsou. 1993. Conformational changes at the active site of creatine kinase at low concentrations of guanidinium chloride. *Biochem. J.* 291:103–107.