

Inhibition of Plasma Membrane Ca^{2+} -ATPase Activity by Volatile Anesthetics

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Background: The precise sites and mechanisms of action of volatile anesthetics remain unknown. Recently, several integral membrane proteins have been suggested as potential targets to which anesthetics can bind at hydrophobic regions. Impairment of cell Ca^{2+} homeostasis has been postulated as one of the possible mechanisms of anesthetic action. To test these hypotheses, the authors selected the human erythrocyte Ca^{2+} -ATPase as a model membrane protein. This enzyme is an integral membrane protein that is instrumental in maintaining Ca^{2+} homeostasis in the cell in which it is the sole Ca^{2+} -transporting system. Thus, any functional alteration of the Ca^{2+} -ATPase by anesthetics may lead to serious perturbations in Ca^{2+} -regulated processes in the cell.

Methods: The Ca^{2+} -ATPase activity was measured as a function of increased concentration of four volatile anesthetics: halothane, isoflurane, enflurane, and desflurane.

Results: All four anesthetics significantly inhibited the Ca^{2+} -ATPase activity in a dose-dependent manner. The half-maximal inhibition occurred at anesthetic concentrations from 0.3 to 0.7 vol% at 37°C , which, except for desflurane, is a clinically relevant concentration range. The greater the clinical potency of the volatile anesthetics studied, the less was the concentration required to inhibit the Ca^{2+} -ATPase activity. The inhibition was less at 25°C than at 37°C , which is consistent with direct interactions of the nonpolar interfaces of the enzyme with the nonpolar portions of the anesthetics.

Conclusions: The authors' findings indicate that the Ca^{2+} -ATPase is a suitable model for investigating the mechanism

of action of volatile anesthetics on the integral membrane protein, and that this inhibition may be specific. (Key words: Anesthetics, volatile: desflurane; enflurane; halothane; isoflurane. Cell: erythrocyte. Enzymes, Ca^{2+} -ATPase: enzyme inactivation. Ions, calcium: cell Ca^{2+} homeostasis.)

THE precise sites and mechanisms of action of volatile anesthetics remain unknown. In recent years, several observations have been published suggesting that general anesthetics may act on specific hydrophobic sites on protein molecules, rather than interact nonspecifically with membrane lipids.¹⁻⁵ It was possible to identify distinct anesthetic binding sites for several soluble proteins, among them bovine serum albumin, luciferase, and protein kinase C.^{1,6,7}† It is interesting that most soluble proteins are not affected by anesthetics at concentrations that induce general anesthesia. Some proteins are inhibited by certain agents, but not by others, indicating that general anesthetics selectively affect certain proteins.³

The many theories developed to explain the mechanism of the action of anesthetics have a common theme; *i.e.*, that the anesthetics alter some component of the membrane, leading to altered function. Thus, an integral plasma membrane protein appears to be a highly probable target for volatile anesthetics. Some integral membrane proteins, such as the acetylcholine receptor-ionophore complex, rhodopsin, and ion-channel proteins, have been suggested as potential sites where general anesthetics could exert their functional effects.⁸⁻¹¹ We have selected an integral membrane protein, the human erythrocyte Ca^{2+} -ATPase, as a model for studies of the mechanism of action of volatile anesthetics on plasma membrane proteins. This enzyme is instrumental in Ca^{2+} homeostasis in the cell, because it is the sole Ca^{2+} -transporting system in the absence of voltage-regulated Ca^{2+} channels, Na/Ca exchanger, or ER type Ca^{2+} -ATPase in human erythrocytes. Thus, any functional alteration of the Ca^{2+} -ATPase by volatile anesthetics may lead to serious perturbations in Ca^{2+} -regulated processes in the cell. Impairment of Ca^{2+} homeostasis in other cells, such as myocytes, has been

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‡Firestone S, Firestone L: Anesthetic alcohols modulate membrane-bound protein kinase-C function (abstract). FASEB J 2:A1381, 1988.

postulated as one of the possible mechanisms of anesthetic action.¹²⁻¹⁵

We developed conditions providing adequate quantities of purified and functional, well characterized Ca^{2+} -ATPase, and established a straightforward assay of the volatile anesthetics' effect on the Ca^{2+} -ATPase activity.¹⁶⁻²³ Indirect methods, such as inactivation studies, had to be applied, because high hydrophobicity of membrane proteins makes measurements of the specific volatile anesthetics binding by ^{19}F -NMR spectroscopy impractical.⁷ To determine whether the Ca^{2+} -ATPase is a suitable model for studies of the mechanism of action of volatile anesthetics on plasma membrane proteins, we investigated the effects of four volatile anesthetics on enzyme activity, characterized the inhibition quantitatively, and compared the inhibitory potency of the anesthetics to their clinical potency. We established that the observed inhibition of the Ca^{2+} -ATPase meets the following criteria for a good model protein: (1) it is dose-dependent, (2) it is reversible, (3) it occurs at clinically relevant concentrations, and (4) the correlation between clinical and inhibitory potency is very good. We compared the inhibition of the purified enzyme with that in the erythrocyte ghost membrane to assure that the effect occurs directly on this particular enzyme. We also used ghost membranes to test the specificity of the anesthetic effect on the Ca^{2+} -ATPase by showing that the Ca^{2+} -independent Mg^{2+} -ATPase present in the membrane is not inhibited by the anesthetics at their clinical concentrations that inhibit the Ca^{2+} -ATPase.

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); and bovine brain calmodulin was from Calbiochem (San Diego, CA). Coupling of bovine calmodulin to Sepharose was performed in accordance with Pharmacia LKB Biotechnology instructions, as described earlier.¹⁸ Thymol-free halothane was obtained from Halocarbon Laboratories (River Edge, NJ); and enflurane (Ethrane), isoflurane (Forane), and desflurane (Suprane) were obtained from Anaquest (Liberty Corner, NJ).

The methods used for erythrocyte membrane ghosts preparation, enzyme purification, determination of protein concentrations, Ca^{2+} concentrations, and Ca^{2+} -ATPase activity assays were described previously.^{17,20} Briefly, the enzyme was studied either in erythrocyte ghost membranes or after purification from the membranes by calmodulin affinity column chromatography in the presence of the nonionic detergent C_{12}E_8 . Total calcium was measured by atomic absorption, and free Ca^{2+} concentrations were calculated from total calcium and EGTA. Ca^{2+} -ATPase activity was determined by measurement of inorganic phosphate production. The activity assay was performed in sealed polypropylene 1.7-ml tubes in a total reaction volume of 100 μl . After the addition of all reagents, immediately after starting the reaction with 3 mM ATP, the volatile anesthetic was delivered to the reaction tube in an air-tight Hamilton syringe, whereupon the tube was sealed and vortexed. The reaction was carried out for 15–30 min at 37° C or 25° C. Samples were terminated with ammonium molybdate/metavanadate at individual times, so the anesthetic was not depleted. Steady state velocities were obtained from plots of inorganic phosphate production, which are linear with time. The aliquots of volatile anesthetics delivered to the assay tube were taken from solutions of saturated volatile anesthetics in reaction mixture, and were prepared daily from the stock of pure volatile anesthetics under nitrogen gas. Except for desflurane solutions, which were prepared at 4° C, all anesthetic solutions were prepared at room temperature. In parallel to the activity assay, tubes were incubated under identical conditions and used for measurements of the effective volatile anesthetic concentrations in the reaction mixture during the activity assay. For concentration measurements, the anesthetic was extracted from the reaction mixture with heptane, and was measured by gas chromatography using a Shimadzu 8A Gas Chromatograph (Columbia, MD) with a glass column with Torapack Q 60/80 mesh packing and a TCD detector.

The concentrations of volatile anesthetics in vol% in gas were calculated from measured coefficients. The following equations were used: (1) vol% in gas (ml of volatile anesthetics vapor/100 ml air) = $[\text{anesthetic}]_{\text{gas}} \times V'$, where $V' = V \times T'/T$ and V' and V = the molar gas volume at temperature T' and at standard condition ($T = 273^\circ \text{K}$), respectively; (2) $[\text{anesthetic}]_{\text{gas}} = [\text{anesthetic}]_{\text{RM}}/\lambda$, where λ = the partition coefficient (RM/gas), RM = the reaction mixture, and $[\text{anesthetic}]$ is defined in mM; and (3) $[\text{anesthetic}]_{\text{gas}}$

§ Blanck TJJ: Personal communication. 1993.

$= C_{GC} \times e_{VA}/MW_{VA}$, where C_{GC} = the concentration of the anesthetic in the reaction mixture as measured by gas chromatography in heptane extract (μ l anesthetic/ml heptane), e_{VA} = the anesthetic density, MW_{VA} = its molecular weight, and VA = volatile anesthetic.

The partition coefficients were measured for each volatile anesthetic at 37° C (and additionally at 25° C for halothane), after 2 h incubation with 30 s vortexing at 15-min intervals. The partition coefficient is defined as the ratio of the amount of volatile anesthetics present in the reaction mixture phase compared with that in the gas phase, the two phases being of equal volume and in equilibrium.²⁴ Our measured values for partition coefficients at 37° C are listed in table 1, together with the literature values used for volatile anesthetic density and the MAC values (minimum alveolar concentration of an agent that produces immobility in 50 percent of those subjects exposed to a noxious stimulus).²⁵ The listed MAC values have been measured at 36–39° C, and are expressed here in vol% in vapor.

The reversibility of the inhibition of the Ca^{2+} -ATPase activity was demonstrated after partial removal of the anesthetic from the reaction mixture by an N_2 purge. Proper controls were run in parallel, including purging the reaction mixture that did not contain anesthetic, and comparing the Ca^{2+} -ATPase activities after purging to the values obtained at standard assay conditions in the sealed tube. The procedure resulted in 40–50% reversibility of inactivation on an estimated 50% removal of halothane.

Standard deviations (SD) and standard errors of the mean (SEM) were used to compare different groups of data. Data are expressed as the mean \pm SEM of independent experiments.

Table 1. Biophysical Properties of Volatile Anesthetics

Volatile Anesthetic	Partition Coefficient	MAC (vol%)	Density (g/ml)
Halothane	0.845 \pm 0.048 (8)	0.75	1.86
Isoflurane	0.680 \pm 0.048 (8)	1.15	1.49
Enflurane	0.751 \pm 0.038 (10)	1.68	1.52
Desflurane	0.297 \pm 0.035 (17)	6.00	1.47

Values are expressed as mean \pm SD; the number of experiments is given in parentheses. Partition coefficients were measured as described in Methods. MAC and density values are from references 5, 31, and 32.

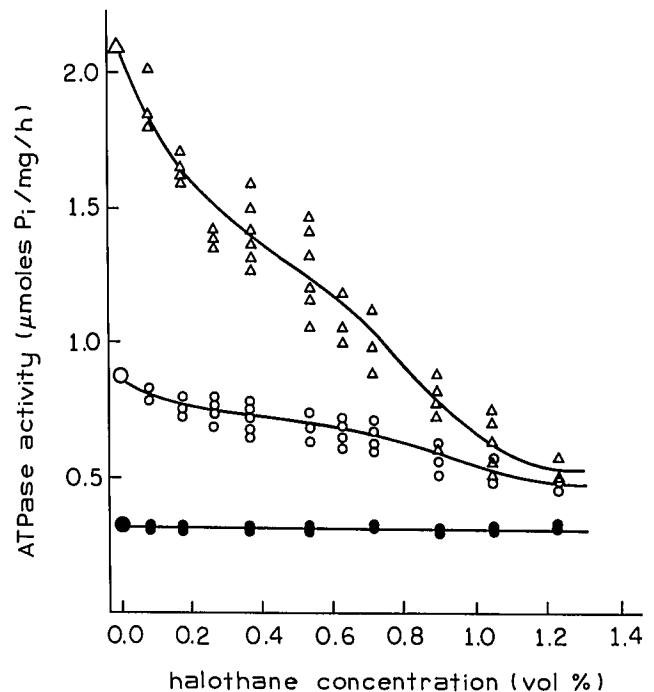


Fig. 1. Inhibition of the Ca^{2+} -ATPase activity in human erythrocyte membrane by halothane. The ATPase activity was assayed as described in the materials and methods section. The reaction mixture contained 50 mM Tris-maleate, pH 7.4, 120 mM KCl, 8 mM $MgCl_2$, and 1 mM EGTA for determination of the Ca^{2+} -independent Mg^{2+} -ATPase activity (\bullet), and, in addition, 17.5 μ M free Ca^{2+} for determination of the Ca^{2+} -dependent activation of the Ca^{2+} -ATPase in the absence (\circ) and presence (Δ) of calmodulin. Protein concentration was 10–12 μ g of erythrocyte membrane and 1 μ g calmodulin in the total reaction volume of 100 μ l. Values represent four to six data points for each halothane concentration.

Results

The Effect of Halothane on Ca^{2+} -ATPase Activity in Erythrocyte Ghost Membranes

As shown in figure 1, halothane causes significant reduction in the Ca^{2+} -ATPase activity both in the absence and presence of calmodulin. Half-maximal inhibition is observed at 0.57–0.85 vol% of halothane. In contrast, the Ca^{2+} -independent Mg^{2+} -ATPase activity present in the erythrocyte membranes is not affected by halothane at these concentrations (fig. 1).

The Effect of Halothane on Ca^{2+} -ATPase Activity in Purified Enzyme

Subsequently, the action of halothane was studied using purified solubilized Ca^{2+} -ATPase preparation to determine whether the demonstrated effect of halo-

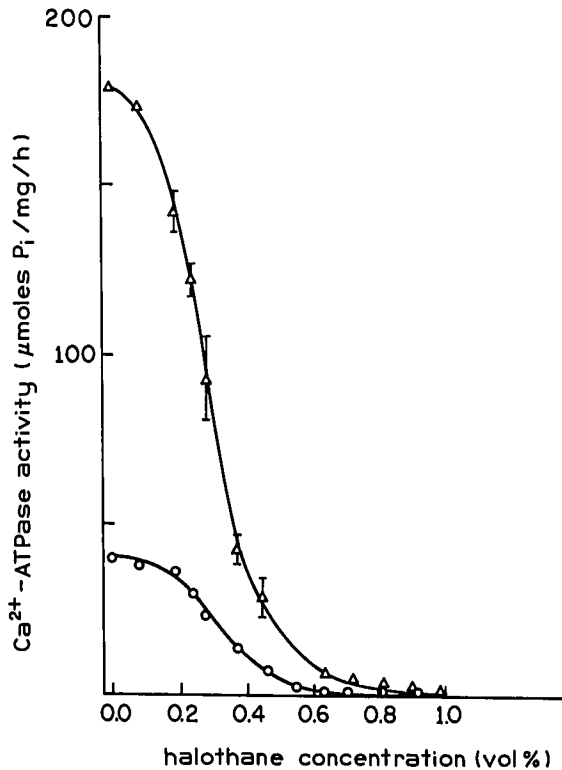


Fig. 2. Inhibition of the Ca^{2+} -ATPase activity in the purified enzyme by halothane in the absence (O) and presence (Δ) of calmodulin. The ATPase activity was assayed as described in the materials and methods section. The reaction mixture contained 50 mM Tris-maleate, pH 7.4, 20 mM KCl, 8 mM MgCl_2 , 150 μM C_{12}E_8 , 1 mM EGTA, 3 mM ATP, and 45 nM (0.6 μg in 100 μl) enzyme. Free Ca^{2+} was at 100 nM. When present, calmodulin was at 40 nM. The error bars indicate SEM of replicates, and are shown when their dimensions exceed those of the symbols.

thane is directly on this enzyme, and not through some other membrane component. As shown in figure 2, the inhibition patterns of the Ca^{2+} -ATPase activity of the purified enzyme, both in the presence and absence of calmodulin, are comparable to those observed in the ghost membrane, in that both activities are inhibited quite dramatically and in a dose-dependent manner. However, the biphasic effect noticeable in ghosts at low anesthetic concentrations is not seen in the purified enzyme, and the half-maximal inactivation in purified enzyme occurs at lower halothane concentrations (0.32–0.34 vol%).

Comparison of the Effect of Four Volatile Anesthetics on the Ca^{2+} -ATPase Activity

To establish whether the inhibition of Ca^{2+} -ATPase activity by halothane is a general property of haloge-

nated volatile anesthetics, we included in the study two other volatile anesthetics commonly used in clinical practice (isoflurane and enflurane) and one that is currently undergoing clinical trials (desflurane). As shown in figure 3 for the calmodulin-dependent Ca^{2+} -ATPase activity, all studied anesthetics inhibit the enzyme in a dose-dependent manner that is similar to halothane. The order of inhibitory potency is a decreasing one, from halothane, to isoflurane, to enflurane, and then to desflurane.

The inhibitory potencies of the volatile anesthetics, when plotted against their corresponding clinical potencies (fig. 4), show that, the higher the MAC value of the volatile anesthetics, the higher the concentration required to inhibit the Ca^{2+} -ATPase activity, both in the presence and absence of calmodulin. As shown by bars in figure 4, although there are some differences in I_{50} values determined for different enzyme preparations, the order of volatile anesthetics potency and the ratio between concentration dependence of the four anesthetics are always the same. The half-maximal inhibition of the calmodulin-dependent and calmodulin-independent activity, as established on several enzyme preparations, occurs at 0.38–0.44 vol% isoflurane, 0.45–0.47 vol% enflurane, and 0.52–0.64 vol% desflurane.

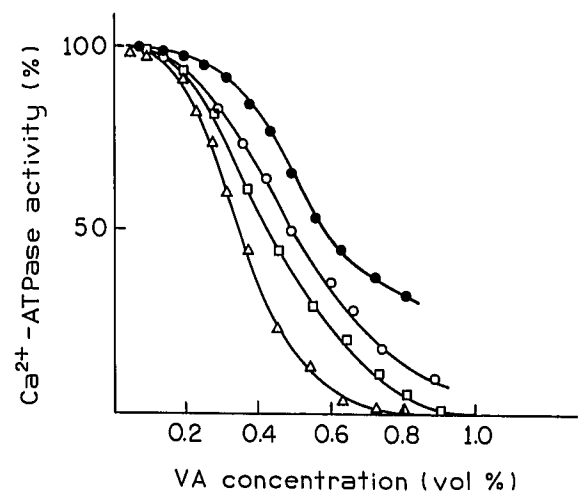


Fig. 3. Concentration dependence of the inhibitory effect of four volatile anesthetics on the Ca^{2+} -ATPase activity in purified enzyme in the presence of calmodulin. The activity assay was performed as described in the materials and methods section and figure 2. Values represent means of triplicate measurements of three to seven separate experiments performed for each volatile anesthetic: halothane (Δ), isoflurane (\square), enflurane (O), and desflurane (\bullet).

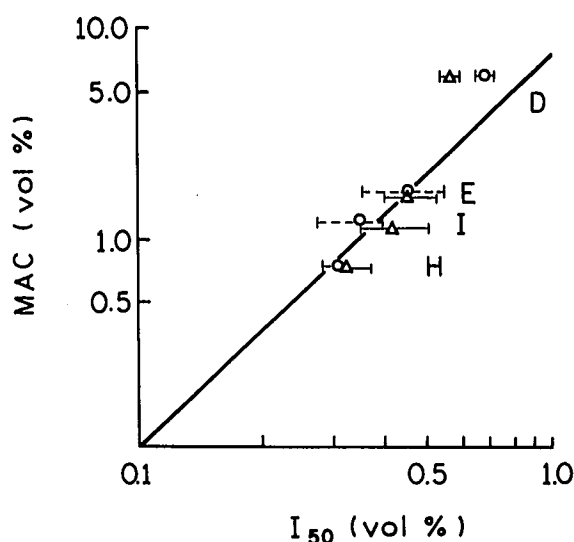


Fig. 4. Correlation between clinical potency of halothane (H), isoflurane (I), enflurane (E), and desflurane (D), and their ability to inhibit the Ca^{2+} -ATPase activity. The clinical potency is expressed in MAC values (in vol%), as described under in the materials and methods section. I_{50} is the concentration of the particular anesthetic (in vol%) that causes 50% inhibition of enzyme activity either in the absence (O) or presence (Δ) of calmodulin. These are averaged values calculated from the data from several experiments. Bars represent the whole range of I_{50} values determined in the absence and presence of calmodulin.

Temperature Dependence of the Inhibition of the Ca^{2+} -ATPase Activity by Volatile Anesthetic

The inhibition patterns of the Ca^{2+} -ATPase activity observed in the presence of halothane at 37°C (fig. 2) were compared with halothane's effect at 25°C to assess the nature of the interactions between volatile anesthetics and enzyme. As shown in figure 5, the inhibition, as expressed in percentage of activity in the absence of volatile anesthetics at the respective temperature, is less at 25°C ($I_{50} = 0.46$ vol%) than at 37°C ($I_{50} = 0.34$ vol%). The ratio of 1.3–1.4 between the extent of inactivation at 25°C and 37°C is the same for the Ca^{2+} -ATPase activity, in both the presence and absence of calmodulin.

Discussion

Four volatile anesthetics have been selected for the study of volatile anesthetic effect on the human erythrocyte Ca^{2+} -ATPase. Three of these belong to a series of halogenated methyl ethyl ether derivatives, while the fourth, halothane, is a two carbon alkane derivative

(fig. 1). The four anesthetics comprise a homologous series with respect to their clinical potency in humans (table 1).

All four anesthetics decrease human erythrocyte Ca^{2+} -ATPase activity. In all cases, this inhibitory effect is exerted in a range of concentrations routinely used in clinical anesthesia at 37°C . The enzyme's sensitivity to the anesthetics appears to be great: the Ca^{2+} -ATPase activity is half inhibited at anesthetic concentrations equal to ≈ 0.3 – 0.4 MAC for enflurane, halothane, and isoflurane, and ≈ 0.13 MAC for desflurane. The reason for the distinctly lower value of the ratio I_{50}/MAC obtained for desflurane is not clear at this time.

The order of inhibitory potency of the four anesthetics shows that, with respect to the concentration required to half-maximally inhibit the Ca^{2+} -ATPase activity, halothane is the most potent. This order is consistent with the reported order of inhibitory potency of halothane, isoflurane, and enflurane on Ca^{2+} transport in other systems. For example, Ca^{2+} uptake by the SR Ca^{2+} -ATPase is inhibited the most by halothane and the least by enflurane.²⁶ In skinned fibers, Ca^{2+} release from the sarcoplasmic reticulum has also been shown to be de-

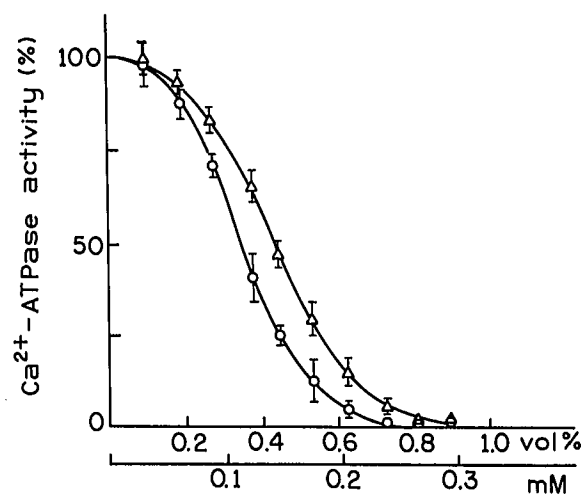


Fig. 5. Comparison of Ca^{2+} -ATPase activity inhibition by halothane at 37°C (O) and 25°C (Δ) in the presence of calmodulin. The ATPase activity was assayed as described in the materials and methods section and figure 2. The data points collected in the presence of halothane are expressed in percentage of the Ca^{2+} -ATPase activity detected in the absence of the anesthetic at the respective temperature. The specific activity at 37°C was $180 \mu\text{moles P}_i \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$, and, at 25°C , it was $75 \mu\text{moles P}_i \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. The measured partition coefficient at 25°C was 1.04 ± 0.44 . The error bars indicate SEM of replicates, and are shown when their dimensions exceeded those of the symbols.

pressed by the volatile anesthetics, with halothane, again, more potent than isoflurane.²⁷ Electrophysiologic studies indicate that the volatile anesthetics depress slow channel mediated Ca^{2+} flux into the myocyte, with halothane being more potent than isoflurane.²⁸ No comparative biochemical studies have been published on desflurane.

The purified, detergent-solubilized Ca^{2+} -ATPase preparation has been repeatedly demonstrated to be very well suited for studies of the Ca^{2+} -ATPase in plasma membrane (for review, see Kosk-Kosicka²²). The current report also shows its appropriateness for studies of the volatile anesthetics' effect on the enzyme in erythrocyte membranes. It also seems highly probable that our findings on this purified enzyme could represent volatile anesthetics' effect on Ca^{2+} -ATPases operating in the plasma membrane of other cells. For example, we observed that halothane inhibits the Ca^{2+} -ATPase activity in crude preparations of dog brain synaptosomal membranes in a manner similar to that demonstrated for the enzyme in human erythrocyte ghosts (unpublished results).

The observed temperature dependence of the inhibition by halothane provides some insight into the nature of the interaction that leads to the enzyme inhibition. The fact that the inhibition of the Ca^{2+} -ATPase by halothane is less at 25° C than at 37° C is consistent with direct interactions of the nonpolar portions of halothane with nonpolar surfaces of the enzyme, because the hydrophobic interactions are weaker at decreased temperature.^{29,30} Weaker interactions at 25° C apparently cause weaker inhibition of the Ca^{2+} -ATPase in both activation pathways, *i.e.*, in the presence and absence of calmodulin. A specific binding of the volatile anesthetics to the hydrophobic regions on the enzyme protein, rather than a membrane bilayer lipid mediated effect, is also in agreement with the lack of change in erythrocyte membrane lipid fluidity measured as the order parameter with 5-, 12-, and 16-doxyl stearic acid in the erythrocyte on exposure to 1 vol% halothane. However, the involvement of a few specific phospholipid molecules cannot be excluded.

In conclusion, our data demonstrate that volatile anesthetics inactivate the Ca^{2+} -ATPase, an enzyme crucial for maintaining Ca^{2+} homeostasis in the cell. It remains to be shown whether this phenomenon is closely re-

lated to the clinical effects of anesthesia. The good correlation between anesthetics' clinical potency and the extent of enzyme inhibition demands attention, especially when the target protein(s) for volatile anesthetics' action has not yet been established.

Our data show that the Ca^{2+} -ATPase is a good model protein to study anesthetic effects. Furthermore, this enzyme is easily assayed, easily purified in sufficient quantities, and has a known primary structure. These properties, along with the presented characteristics of enzyme inhibition by volatile anesthetics, make the Ca^{2+} -ATPase an excellent model in which to perform biochemical studies of anesthetic effects on membrane proteins.

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