Hexanol and Lidocaine Affect the Oligomeric State of the Ca-ATPase of Sarcoplasmic Reticulum†

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ABSTRACT: Hexanol at 7 °C stimulates the activity of the Ca-ATPase of sarcoplasmic reticulum (SR). Time-resolved phosphorescence spectroscopy studies of SR whose Ca-ATPase is covalently labeled with erythrosin isothiocyanate (ERITC) indicate that at 7 °C hexanol (1) causes a concentration-dependent increase in the rate of decay of phosphorescence anisotropy, (2) causes larger oligomers of Ca-ATPase to dissociate into smaller oligomers, and (3) increases the rotational mobility of Ca-ATPase in all its oligomeric states. Electron paramagnetic resonance (EPR) spectroscopy of spin-labeled stearic acid (SASL) in SR suggests that at 7 °C hexanol diminishes the fraction of SR lipids in the boundary lipid domain and disorders and fluidizes both the boundary lipid and the unrestricted lipid domain. In protein-free liposomes of extracted SR lipids hexanol increases fluidity and decreases order to a greater extent near the center of the lipid bilayer than near the polar head groups. At 25 °C hexanol has biphasic effects on Ca-ATPase activity: at 10 and 20 mM hexanol increases activity, but at 30 mM and especially at 40 mM there is inhibition of Ca-ATPase activity. The influence of hexanol at 25 °C on the oligomeric state of Ca-ATPase is also biphasic. At 10 and 20 mM, hexanol promotes the dissociation of larger oligomers into smaller ones, whereas at higher concentrations, 30 and 40 mM, hexanol causes larger oligomers to be formed from smaller ones. Lidocaine at 25 °C inhibits Ca-ATPase activity and causes dramatic slowing of the decay of phosphorescence anisotropy of ERITC-labeled SR by causing the formation of larger oligomers of Ca-ATPase from smaller ones. In protein-free liposomes of SR lipids at 25 °C, lidocaine disorders and fluidizes the acyl chains near the center of the bilayer (as did hexanol), but has opposite effects near the polar head groups. *The opposite effects of hexanol and lidocaine on the oligomeric state of the SR Ca-ATPase provide a new molecular explanation for the opposite effects of hexanol and lidocaine on the activity of the Ca-ATPase. We conclude that the biphasic effects of hexanol on the activity of Ca-ATPase can be accounted for by biphasic effects of hexanol on the oligomeric state of the Ca-ATPase.* This study supports the view that anesthetics can alter interactions between membrane proteins.

Numerous studies have reported effects of general and local anesthetics on the function and physical properties of biological membranes. These studies have focused on two areas: the effects of anesthetics on the physical properties of membranes and the effects of anesthetics on membrane proteins (Seeman, 1972; Covino et al., 1985; Firestone, 1986; Roth & Miller, 1986). Membrane properties studied include the order and fluidity of membrane lipids, phase changes in the membrane, boundary lipid effects, and alterations of protein−protein interactions (Bigelow & Thomas, 1987; Taraschi et al., 1991; Trudell, 1991; Fraser et al., 1990). The study of anesthetic effects on biological membranes may not only provide insight into the molecular mechanisms of anesthetic action, but also helps to elucidate mechanisms of regulation of membrane proteins.

Because of the extensive characterization of the influence of membrane properties on the activity of the sarcoplasmic reticulum (SR[1]) Ca-ATPase (reviewed by Thomas and Mahaney, 1993), the SR is a favorable system for investigating the influence of general and local anesthetics on the functions of biological membranes. Effects of alterations in SR lipid fluidity, Ca-ATPase rotational mobility, and the oligomeric state of the Ca-ATPase on the function of the Ca-ATPase have been reported (Squier et al., 1988a; Squier et al., 1988b; Birmachu & Thomas, 1990; Karon & Thomas, 1993). Changes in lipid physical parameters, such as SR lipid order and fluidity (Squier et al., 1988b) and boundary lipid characteristics (Bigelow & Thomas, 1987), have been correlated with changes in protein physical parameters such as oligomeric state (Birmachu & Thomas, 1990) and rotational mobility of the Ca-ATPase (Bigelow et al., 1986). Effects of other perturbations, such as temperature (Bigelow et al., 1986; Thomas & Karon, 1994), membrane thickness (Cornea & Thomas, 1994), diethyl ether (Bigelow & Thomas,

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[1] Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ED_{50}, dose required to produce half of maximum effect; EPR, electron paramagnetic resonance, ERITC, erythrosin 5-isothiocyanate; ERITC-SR, SR labeled with ERITC; MAC, minimal anesthetic concentration; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, β-nicotinamide adenine dinucleotide, reduced form; PC, phosphatidylcholine; PEP, phospho(enol)pyruvate; 5-SASL [5-[(4,4-dimethyloxazolidinyl)oxy]stearic acid]; 14-SASL [14-[(4,4-dimethyloxazolidinyl)oxy]stearic acid]; SR, sarcoplasmic reticulum; TPA, time-resolved phosphorescence anisotropy.

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1987), *n*-alcohols (Lopes & Louro, 1991), and melittin (Mahaney & Thomas, 1991; Voss et al., 1991; Mahaney et al., 1992) on lipid−protein interactions and Ca-ATPase activity have been studied. The effects of general and local anesthetics on the Ca-ATPase of SR of skeletal muscle has also been studied.

General Anesthetics. Diethyl ether, at concentrations from 2 to 8% (v/v), markedly enhances the ATPase activity and rate of active Ca^{2+} transport by Ca-ATPase in skeletal SR membrane vesicles (Inesi et al., 1967; Salama & Scarpa, 1980; Bigelow & Thomas, 1987). In the same range of concentrations, ether also disorders and fluidizes the acyl chain region of the SR membrane phospholipids, especially in the lipid−protein interface, and increases the average rate of rotational diffusion of the Ca-ATPase in the membrane (Bigelow & Thomas, 1987; Squier et al., 1988b).

There are about a dozen studies of the effect of halothane on active Ca^{2+} uptake by skeletal SR. The effects depend on the concentration of halothane used: at concentrations below 3−4 MAC (minimal anesthetic concentration[2]) halothane most often stimulates Ca^{2+} uptake by skeletal SR (Nelson et al., 1972); at higher concentrations halothane inhibits Ca^{2+} uptake (Dhalla et al., 1972; Gronert, et al., 1979; Nelson & Sweo, 1988). A recent study from this laboratory (Karon & Thomas, 1993) concludes that above 20 °C the stimulation of Ca-ATPase activity by halothane is due to enhancing the SR membrane fluidity and thus the rotational mobility of the Ca-ATPase, while below 20 °C, halothane stimulates Ca-ATPase activity partly by causing larger, less active oligomers of Ca-ATPase to dissociate into more active smaller oligomers.

n-Alcohols are general anesthetics. *n*-Alkanols (from methanol to heptanol) have been reported to have biphasic effects on the activity of Ca-ATPase of skeletal SR (Kondo & Kasai, 1973; Almeida et al., 1986; Melgunov et al., 1987). As is the case with halothane, at lower concentrations the alcohols markedly stimulate Ca-ATPase activity; at higher concentrations Ca-ATPase activity is inhibited. The *n*-alkanols, from butanol to pentanol, increase the fluidity of SR membrane lipids (Zavoico & Kutchai, 1980). *n*-Alcohols were reported to diminish the fraction of SR lipids in the boundary lipid of the Ca-ATPase (Lopes & Louro, 1991).

Local Anesthetics. Local anesthetics have been reported to inhibit Ca^{2+} uptake and Ca-ATPase activity in skeletal SR (Johnson & Inesi, 1969; Boland et al., 1975; Suko et al., 1976). Local anesthetics appear to diminish the affinity of the Ca-ATPase for Ca^{2+} and to decrease the rate of formation of the phosphorylated intermediate of the Ca-ATPase of skeletal muscle (Suko et al., 1976).

The effects of local anesthetics on the activity of Ca-ATPase of skeletal SR are generally inhibitory, while general anesthetics at low concentrations, by contrast, stimulate Ca-ATPase activity.

Contribution of the Present Study. A major aim of this study is to understand the mechanisms whereby general and local anesthetics produce opposite effects on activity of the Ca-ATPase of skeletal SR. Previous studies from this laboratory suggest that anesthetics may influence the activity of Ca-ATPase by affecting the rotational mobility of the Ca-ATPase and/or by altering the oligomeric state of the protein (Birmachu & Thomas, 1990; Karon & Thomas, 1993; Kutchai et al., 1993). The present study compares the effects of a general anesthetic (*n*-hexanol) with those of a local anesthetic (lidocaine) on Ca-ATPase activity and SR molecular dynamics in order to better understand the opposite effects of these agents on Ca-ATPase activity.

Two phenomena are described in this paper that have not previously been reported: (1) the finding that a local anesthetic (lidocaine) causes the formation of larger oligomers of Ca-ATPase and (2) the observation that a general anesthetic (hexanol) has biphasic effects on the oligomeric state of the Ca-ATPase, promoting the formation of small oligomers at low hexanol concentration, but enhancing the formation of larger oligomers at higher hexanol concentration. Our findings provide a new molecular interpretation for the opposite effects of general and local anesthetics and the biphasic effects of general anesthetics on the activity of the Ca-ATPase in terms of the effects of these compounds on the oligomeric state of the Ca-ATPase.

MATERIALS AND METHODS

Reagents and Solutions. Erythrosin-5-isothiocyanate (ERITC) was obtained from Molecular Probes, Inc. (Eugene, OR) and stored in dimethylformamide in liquid N_2. MOPS, Na_2ATP, catalase, glucose, glucose oxidase type IX, PEP, pyruvate kinase, lactate dehydrogenase, and lidocaine were obtained from Sigma Chemical Co. (St. Louis, MO). *n*-Hexanol (99%) and 5-SASL were obtained from Aldrich Chemical Co. (Milwaukee, WI). 14-SASL was the kind gift of Dr. Anthony Watts. Assays were performed in a *standard buffer*: 60 mM KCl, 25 mM MOPS (pH 7.0), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, except where noted.

Preparations and Assays. SR vesicles were prepared from the fast twitch skeletal muscle of New Zealand white rabbits (Fernandez et al., 1980). The vesicles were purified on a discontinuous sucrose gradient (Birmachu et al., 1989) to remove junctional SR containing calcium release proteins. All preparation was done at 4 °C. SR pellets were resuspended in 0.3 M sucrose, 20 mM MOPS (pH 7.0), rapidly frozen in 4 mg aliquots, and stored in liquid N_2 until use. SR lipids were extracted by a modification (Hidalgo et al., 1976) of the method of Folch et al. (1957), using N_2-saturated solvents to prevent oxidation. The lipids were stored in chloroform−methanol (2:1) under nitrogen at −20 °C.

The SR Ca-ATPase activity was estimated with a continuous ATPase assay using an ATP-regenerating system (Madden et al., 1979). Assays were performed in buffer in the presence of phosphoenolpyruvate, NADH, pyruvate kinase, lactate dehydrogenase, and ATP as described in the relevant figure legends. The assay mix was calibrated by adding aliquots of 10 mM ADP and observing the change in absorbance at 340 nm. SR (20 μg/mL) was added to the assay mix and preincubated with hexanol or lidocaine. Then ATP (1 mM was added to begin the assay. The rate of change of absorbance of NADH at 340 nm was recorded to estimate the rate of ATP hydrolysis. The Ca^{2+} ionophore A23187 (0.2 μg/mL) was added to allow the Ca-ATPase activity to be measured in the absence of a Ca^{2+} gradient, so that vesicle leakiness produced by the addition of an anesthetic will not affect the activity measured (Bigelow &

[2] MAC (minimal anesthetic concentration) is the concentration of the anesthetic required to produce lack of a reflex response to a skin incision in 50% of the subjects. In man this corresponds to a level of halothane in the blood of about 0.25 mM.

Thomas, 1987). In some cases Ca-ATPase enzymatic activity was also determined in the absence of A23187. The anesthetics, at the levels used, did not significantly affect the ATP-regenerating system.

SR protein concentrations were estimated by the biuret method (Gornall et al., 1949) using bovine serum albumin as standard.

Preparation of Samples for Time-Resolved Phosphorescence Anisotropy (TPA) Determinations. Ca-ATPase was specifically labeled at lysine 515 with ERITC as described by Birmachu and Thomas (1990). The labeled SR (ERITC-SR) was suspended at 10 mg protein/mL in 30 mM MOPS (pH 7.0), 0.3 M sucrose, 0.1 mM $CaCl_2$ and kept in the dark on ice until use. Oxygen was removed from the TPA samples with 100 μg/mL of glucose oxidase, 15 μg/mL of catalase, and 5 mg/mL of glucose (Eads et al., 1984) in the standard buffer in the phosphorescence cuvette. The cuvette was flushed with argon, and then deoxygenation occurred in the sealed cuvette containing 0.2−0.3 mg/mL of SR protein for at least 10 min prior to phosphorescence data collection. When indicated, the standard buffer contained *n*-hexanol or lidocaine at the specified concentration. The time the ERITC-SR was in the presence of the anesthetic before data collection was begun is termed the *preincubation time*.

Instrumentation for TPA. The instrumentation for TPA was previously described by Ludescher and Thomas (1988). The phosphorescence anisotropy decay $r(t)$ is given by

$$r(t) = \frac{I_{vv}(t) - GI_{vh}(t)}{I_{vv}(t) + 2GI_{vh}(t)} \tag{1}$$

where $I_{vv}(t)$ and $I_{vh}(t)$ are obtained by signal-averaging the time-dependent phosphorescence decays, with a single detector and a polarizer that alternates between the vertical $[I_{vv}(t)]$ and horizontal $[I_{vh}(t)]$ positions every 2000 laser pulses. The laser repetition rate was 200 Hz; a typical $r(t)$ acquisition required about 4.5 min to complete 10 loops, or cycles, of 4000 laser pulses each (2000 in each polarizer orientation). G is an instrumental correction factor, determined by measuring the anisotropy of free dye in solution under similar experimental conditions.

Analysis of TPA Data. It has been shown previously (Birmachu & Thomas, 1990) that the TPA decay of ERITC-SR is dominated by the uniaxial rotation of the labeled Ca-ATPase about an axis normal to the bilayer. In theory, this should result in biexponential anisotropy decay component for each distinct rotating species (Kinosita et al., 1984; reviewed by Thomas, 1986). However, Birmach and Thomas (1990) have shown that for ERITC-SR, only one of the two exponentials contributes significantly, and the TPA decay is described accurately by

$$\frac{r(t)}{r_o} = \sum_{i=1}^{n} A_i e^{-t/\phi_i} + A_\infty \tag{2}$$

where ϕ_i is the rotational correlation time (inversely proportional to the rotational diffusion coefficient D_{mi} for a given rotating species), A_i are the normalized amplitudes (r_i/r_o), and r_o is the initial anisotropy, $r_o = r(0) = \sum r_i(0) + r_\infty$. A_∞ is the normalized residual anisotropy (r_∞/r_o).

$$A_\infty = A_{\infty o} + f_I(1 - A_{\infty o}) \tag{3}$$

where f_I is the fraction of proteins that is immobile on the observed time scale and $A_{\infty o}$ is the residual anisotropy of a reference sample for which $f_I = 0$. $A_{\infty o}$ describes the extent to which the probe's motion is restricted in angular amplitude, due to the fixed angles θ_a and θ_e of the probe's absorption and emission transition moments, respectively, relative to the membrane normal

$$A_{\infty o} = \frac{P_2(\cos\theta_a)P_2(\cos\theta_e)}{P_2(\cos\delta)} \tag{4}$$

where $P_2(x) = (3x^2 - 1)/2$, and δ is the angle between the absorption and emission transition dipoles (Lipari &Szabo, 1980).

The fraction of probes with a correlation time ϕ_i is thus given by $f_i = A_i/(1 - A_{\infty o})$.

Equation 2 was fit to the anisotropy decay data using a Marquardt−Levenberg fitting routine (Birmachu & Thomas, 1990). The goodness-of-fit was evaluated by comparing χ^2 values for the multiexponential fits and by comparing plots of the residuals (the difference between the measured and the calculated values). Our TPA data was fit better by three exponential components than by two, but no significant improvement in χ^2 was achieved by using four exponential terms. Thus, we routinely used eq 2 with $n = 3$ to fit our data.

The Saffman−Delbrück equation describes the rotational diffusion coefficient (D_m) for uniaxial rotation of a cylindrical membrane protein rotating about an axis normal to the plane of the membrane, expressed as a function of the membrane lipid viscosity (η), temperature (T), and the effective radius (a) of the portion of the protein in the bilayer (Saffman & Delbrück, 1975)

$$D_m = kT/(4\pi a^2 h\eta) \propto 1/\phi \tag{5}$$

where h is the thickness of the hydrocarbon phase of the lipid bilayer and k is Boltzmann's constant. Thus, the rotational mobility (D_m), inversely proportional to the rotational correlation time (ϕ), should be proportional to the lipid fluidity (T/η) and inversely proportional to the transmembrane cross-sectional area (πa^2) of the rotating protein. Increasing lipid fluidity or decreasing protein size (e.g., formation of smaller oligomers) should result in increased rotational mobility. These relationships between protein size and lipid fluidity and protein rotational mobility are supported by previous studies on the Ca-ATPase using saturation transfer EPR (Squier et al., 1988a, 1988b) and phosphorescence anisotropy (Birmachu & Thomas, 1990) and by studies of the rotational mobility of bacteriorhodopsin (Cherry & Godfrey, 1981). Birmachu and Thomas (1990) showed that rotational correlation times and normalized amplitudes obtained by fitting TPA decays to eq 2 can be used to estimate the sizes and rotational rates of Ca-ATPase oligomers in SR vesicles.

Spin Labeling and Sample Preparation. Lipid hydrocarbon chain rotational mobility was measured using two stearic acid spin labeled probes designated 5-SASL and 14-SASL. Prior to incorporation into SR, the spin label was diluted from a dimethylformamide stock solution into ethanol (due to the greater miscibility of ethanol with water), usually to 10 mM. To incorporate 5- or 14-SASL into SR, the spin label was added to the SR membranes (25 mg/mL in 0.3 M

sucrose, 20 mM MOPS, pH 7.0), and the sample was vortexed well, diluted by a factor of 10 with experimental buffer, and pelleted in a centrifuge to remove any aqueous label. Incorporation of 5- or 14-SASL into protein-free lipid samples (aqueous dispersions) was accomplished by adding the spin label to the extracted lipids in a chloroform–methanol (2:1 v/v) mixture prior to drying with nitrogen. All samples contained 1 mol % spin label, and sample concentrations were kept sufficiently high (>50 mg/mL of SR; >50 mM SR lipids) to minimize the spectral contribution from unbound, aqueous labels. Labeling of SR was carried out at 4 °C, while labeling of SR lipids was carried out at 25 °C.

SR membrane samples containing anesthetic were prepared by the addition of 2 mg of spin-labeled SR to standard buffer containing *n*-hexanol or lidocaine at the specified concentration (final volume 1 mL). The anesthetic-containing SR membranes were pelleted in Beckman TL-100 table top ultracentrifuge to concentrate the vesicles in preparation for EPR analysis. Control samples without anesthetic were treated identically, but substituting buffer for the anesthetic solution.

Protein-free SR lipid samples (which consisted of aqueous dispersions of extracted SR lipids) containing anesthetic were prepared by adding the anesthetic directly to the rehydrated lipids under vortex, followed by additional vortexing. The spectra from the extracted lipids all consisted of a single axially anisotropic component that is characteristic of lipid spin labels in fluid bilayers; none of the spectra contained evidence of anesthetic-induced spectral heterogeneity, which would arise from the formation of anesthetic-lipid probe micelles in our samples (Mahaney & Thomas, 1991).

EPR Spectroscopy. EPR spectra were acquired using a Bruker ESP-300 spectrometer equipped with a Bruker ER4201 cavity and digitized with the spectrometer's built-in microcomputer using Bruker OS-9-compatible ESP 1620 spectral acquisition software. Spectra were downloaded to an IBM-compatible microcomputer and analyzed using software developed in our laboratory by R. L. H. Bennett. Conventional (V_1) EPR was used to detect submicrosecond motions of the lipid spin labels. V_1 spectra were obtained using 100 kHz field modulation with a peak-to-peak modulation amplitude of 2 G, using a nonsaturating microwave field intensity (H_1) of 0.07 G (Squier & Thomas, 1986; Mahaney et al., 1992). Samples were contained in 50 μL glass capillaries. Sample temperature was controlled to within 0.5 °C with a Bruker ER 4111VT-1003 variable temperature controller. Sample temperature was monitored with a Sensortek Bat-21 digital thermometer using an IT-21 thermocouple probe inserted into the top of the sample capillary, such that it did not interfere with spectral acquisition.

EPR Spectral Analysis. EPR spectra of 14-SASL in SR membranes contain contributions from two distinct motional populations of probes (reviewed by Hidalgo (1985), Marsh (1985), and Bigelow and Thomas (1987)). One component corresponds to labels in a fluid lipid environment, similar to that of spectra obtained from labels in aqueous dispersions of extracted lipids. The other component, which is most evident in the outer wings of the spectra, corresponds to labels in a considerably more restricted environment. This component has been shown to arise from labels whose motion is restricted by interaction with the integral membrane proteins of SR (Marsh, 1985). These composite spectra were resolved into single component spectra by subtraction [cf. Figure 1 of Mahaney et al. (1992)], essentially as described by Marsh (1982). Difference spectra corresponding to the motionally-restricted component of SR composite spectra at low temperatures (0–5 °C) were virtually free from irregularities caused by spectral mismatch during the subtractions. Subtraction of composite spectra at hexanol concentrations above 20 mM was severely hampered due to lack of adequate matching between the fluid component of the composite and any model spectrum of extracted SR lipids. Such mismatch resulted in large spectral irregularities in the fluid region of the restricted-component difference spectra, which made accurate selection of the restricted endpoint nearly impossible. This is probably due to hexanol-enhanced exchange between fluid and motionally restricted components in the membrane, which partially averages the two spectral components and thus prevents their precise resolution (cf. Davoust and Devaux, 1982). Therefore, while quantitative analysis was carried out for composite spectra obtained at each hexanol concentration studied, it should be kept in mind that the analysis done at 30 mM hexanol is less reliable than that at lower hexanol levels.

Single-component spectra were analyzed by measuring the inner ($2T_\perp'$) and outer ($2T_\parallel'$) spectral splittings, where resolved (Squier and Thomas, 1989), which are sensitive both to the rotational amplitude and rotational rate of the spin label (Moser et al., 1989), and the half-width at half-height of the low-field peak (Δ_L), which is sensitive mainly to the rate of motion of the spin label (Marsh, 1981). The effective order parameter (S) of the single-component spectra was determined by the expression (Gaffney, 1976)

$$S = \frac{T_o - T_\perp'}{T_o - T_\perp} = \frac{T_\parallel' - T_o}{T_\parallel - T_o} \tag{6}$$

where T_o is the isotropic hyperfine splitting constant in the absence of anisotropic effects, and $T_\perp$ and $T_\parallel$ are the minimum and maximum values, respectively, of the hyperfine tensor for an axially symmetric system (e.g., a lipid bilayer). The values of T_o, $T_\perp$, and $T_\parallel$ used in this study were 14.3 ± 0.2 G, 6.3 ± 0.3 G, and 33.5 ± 0.4 G, respectively, as previously determined by Squier and Thomas (1989).

To facilitate direct comparisons between anesthetic-induced changes in SR lipid hydrocarbon chain dynamics and protein rotational mobility measured by TPA, the effective lipid fluidity parameter, T/η, was used (Squier et al., 1988b)

$$S = -0.42 \log\left[\frac{T}{\eta}\right] + 0.56 \tag{7}$$

where S is the apparent order parameter of the single-component spectrum (eq 6) and η is the effective lipid viscosity in the vicinity of the label. This measurement of lipid fluidity (T/η) agrees quantitatively with the hydrodynamic theory of Saffman and Delbrück (1975), which predicts that the rotational mobility (diffusion coefficient D_m) of an integral membrane protein about the membrane normal should be proportional to T/η (eq 5). The validity of this EPR-based fluidity measurement is supported by the finding that the rotational mobility (inverse correlation time) of the Ca-ATPase, as measured either by ST-EPR (Squier et al.,

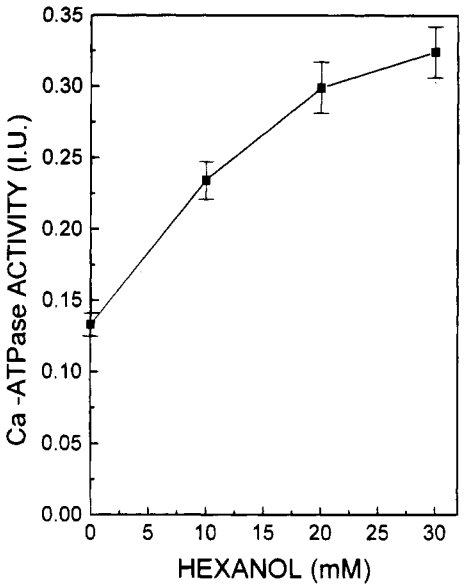

FIGURE 1: Influence of *n*-hexanol on the Ca-ATPase activity of skeletal SR determined as described in the Materials and Methods at 7 °C and pH 7.0 in the presence of 0.2 μg/mL of A23187. SR (20 μg of protein) was assayed in a 1 mL volume of standard buffer with 1 mM Na_2ATP, 0.2 mM PEP, 0.2 mM NADH, 8 units/mL of pyruvate kinase, and 20 units/mL of lactate dehydrogenase.

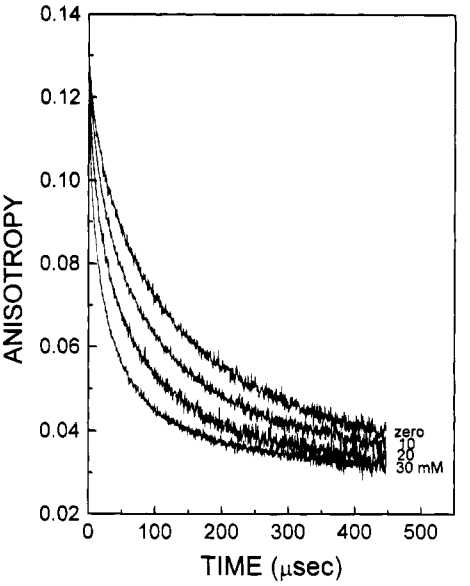

FIGURE 2: Time-resolved phosphorescence anisotropy of ERITC-SR at 7 °C in standard buffer in the absence of *n*-hexanol and in the presence of 10, 20, or 30 mM hexanol. Each curve is the average of at least five separate data aquisition runs.

1988b) or phosphorescence anisotropy (Birmachu & Thomas, 1990), agrees quantitatively with the predictions of eq 5, as long as eq 7 is used to define T/η. Thus, this fluidity parameter, along with eq 5, can be used to determine whether anesthetic-induced changes in Ca-ATPase rotational mobility are due to changes in the membrane lipid fluidity and/or the order of membrane lipids or to changes in the size (oligomeric state) of the rotating proteins.

Changes in the parameters $2T_{\parallel}'$ and $2T_{\perp}'$ and Δ_L in the single-component spectra cannot be related linearly to the changes in lipid chain mobility because of the complications introduced by slow motional effects and inhomogeneous broadening, respectively. To facilitate comparison of the effects of anesthetics on these and other parameters, an anesthetic-induced change in a given parameter is converted to an "effective temperature change" (cf. Mahaney et al., 1992), defined as the temperature change required to produce the same parameter value in a control sample without anesthetic.

RESULTS

Effects of n-Hexanol at 7 °C

Effects of n-Hexanol at 7 °C on Ca-ATPase Activity. Figure 1 shows that *n*-hexanol causes a concentration-dependent increase in the maximal activity (activity in the presence of A23187) of Ca-ATPase. The Ca-ATPase activity in the presence of 30 mM hexanol in 2.44 times the activity in the absence of hexanol. Diethyl ether (Birmachu & Thomas, 1990) and halothane (Karon & Thomas, 1993) were also found, at temperatures near 7 °C, to stimulate Ca-ATPase activity monotonically with increasing concentration of the anesthetic.

Effects of n-Hexanol at 7 °C on Phosphorescence Anisotropy. Figure 2 shows the phosphorescence anisotropy decay of ERITC-labeled SR in the absence of *n*-hexanol and in the presence of 10, 20, or 30 mM hexanol in the standard buffer solution at 7 °C. *n*-Hexanol *increases* the rate of decay of anisotropy in a concentration-dependent manner.

Table 1: Influence of *n*-Hexanol at 7 °C on Calculated Rotational Correlation Times, in μs ± Standard Error, Obtained by Fitting Eq 2 to the Data of Figure 2[a]

[hexanol] (mM)	ϕ_1	ϕ_2	ϕ_3
0	12.7 ± 1.	70.4 ± 6.1	265 ± 12
10	11.5 ± 1.3	61.0 ± 4.6	242 ± 17
20	8.4 ± 1.1	50.3 ± 5.3	205 ± 18
30	6.8 ± 0.7	32.5 ± 2.3	154 ± 10

[a] Shown are the average parameter values obtained by fitting eq 2 to at least five individual data acquisition runs.

The results of Figure 2 could be due (a) to hexanol causing an increased rate of rotation of monomers and oligomers of Ca-ATPase in the SR membrane, (b) to hexanol promoting the conversion of larger oligomers of Ca-ATPase to smaller, more rapidly-rotating oligomers, or (c) to a combination of these two effects. Fitting eq 2 with three time-dependent components to the phosphorescence anisotropy decay data yields estimates of ϕ_1, ϕ_2, and ϕ_3, the rotational correlation times of the smallest rotating species, the intermediate-sized rotating species, and the largest rotating species, respectively. The fitting procedure also yields estimates of A_1, A_2, and A_3, the amplitudes or apparent mole fractions of the smallest, the intermediate, and the largest rotating species, respectively, and A_∞, which partly reflects the apparent mole fraction of species that are too large to rotate appreciably on the time scale of the experiment (eq 3 and Birmachu & Thomas, 1990).

Rotational Correlation Times. Fitting eq 2 with three exponential components to the individual tracings that were averaged in Figure 2 gives the rotational correlation times shown in Table 1. Hexanol decreases the rotational correlation times (increases rotational rates) of all three rotating components.

Amplitudes. Fitting eq 2 to the data of Figure 2 also yields amplitudes (apparent mole fractions) for the three rotating components and an estimate of A_∞. As shown in Figure 3

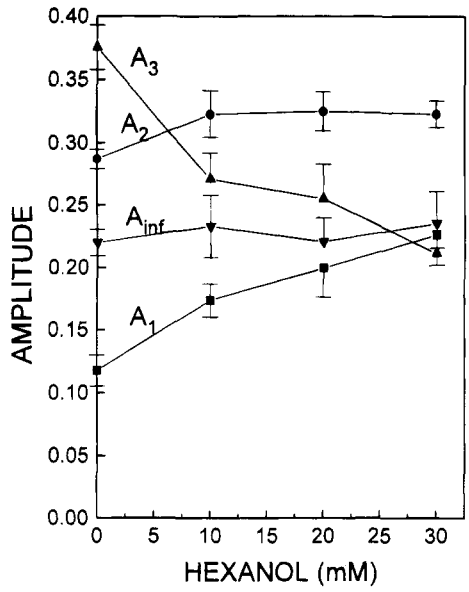

FIGURE 3: Influence of *n*-hexanol at 7 °C on the oligomeric state of Ca-ATPase. Normalized amplitudes (±standard error) result from fitting eq 2 to the data of Figure 2.

the effect of *n*-hexanol is to reduce the magnitude of A_3 (largest rotating oligomers), with a large increase in A_1 (smallest oligomers) and a smaller increase in A_2 (intermediate-sized oligomers). There is no significant change in A_∞ with increasing hexanol. *These data indicate that n-hexanol at 7 °C promotes the dissociation of the largest class of rotating oligomers of Ca-ATPase into the fastest-rotating and intermediate-rotating oligomers.*

At 7 °C treatment of SR with *n*-hexanol results in enhanced proportions of the smaller classes of rotating oligomers of Ca-ATPase, with a decrease in the proportion of the largest rotating oligomers, and in increased rotational rates of all classes of Ca-ATPase oligomers. Similar effects were reported at comparable temperatures with diethyl ether (Birmachu & Thomas, 1990) and with halothane (Karon & Thomas, 1993). *Thus, three different classes of general anesthetics have similar effects on the activity and oligomeric state of the Ca-ATPase at about 7 °C. Our results are consistent with the interpretation that the activity of the Ca-ATPase is enhanced by treatments that promote the formation of smaller oligomers of Ca-ATPase and by treatments that increase the rotational mobility of the Ca-ATPase in the SR membrane.*

Effects of Hexanol at 7 °C on 14-SASL in SR Membranes. EPR spectra of 14-SASL incorporated into SR membranes, with and without 10 mM hexanol, are shown in Figure 4, left panel. The two components of the EPR spectra of 14-SASL can only be resolved at relatively low temperatures. Our interest in investigating the influence of anesthetics on the two populations of membrane lipids was the primary motivation of conducting these experiments, and the TPA experiments, at the unphysiological temperature of 7 °C. As previously demonstrated in SR membranes (Mahaney et al., 1992; Bigelow & Thomas, 1987), the restricted component arises from lipids interacting directly with the integral membrane proteins. Incubation of SR membranes with hexanol affects the spectrum in a manner similar to increasing the sample temperature; i.e., with the progressive addition of hexanol (10, 20, and 30 mM), the inner spectral features (due primarily to fluid lipids) become more narrow and

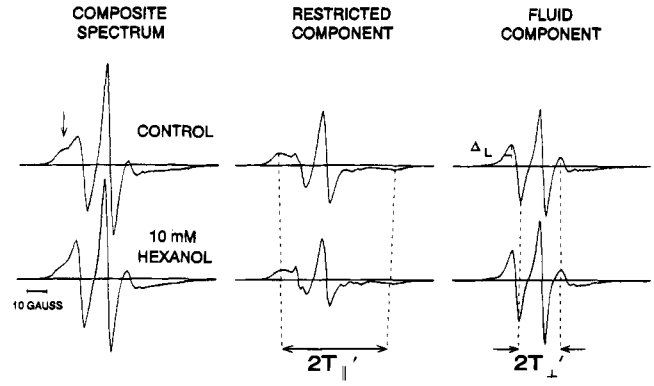

FIGURE 4: Effect of hexanol on the conventional EPR spectrum of 14-SASL incorporated into SR membranes at 7 °C in standard buffer. The arrow (upper left spectrum) indicates the presence of the restricted population of probes in the composite spectra (left). Hexanol-containing SR membranes were incubated with 10 mM hexanol in standard buffer for 15 min at 7 °C, and prepared for EPR analysis as described in the Materials and Methods. Control SR membranes without added hexanol were treated identically, substituting buffer for hexanol. Composite spectra were separated into restricted (center) and mobile (right) components and analyzed as described in the Materials and Methods. Baselines represent 100 G.

Table 2: Influence of *n*-Hexanol on the EPR Spectral Parameters and Derived Quantities of 14-SASL in SR Membranes at 7 °C

	[hexanol] (mM)			
	0	10	20	30
	Restricted Component			
fraction (f_R)	0.64 ± 0.03	0.60 ± 0.01	0.58 ± 0.02	0.57 ± 0.04
$2T_{\|\|}'$	58.3 ± 0.20	57.9 ± 0.3	57.3 ± 0.2	57.4 ± 0.3
ΔL	4.2 ± 0.2	4.3 ± 0.15	4.4 ± 0.1	4.1 ± 0.1
S	0.77 ± .01	0.76 ± .01	0.75 ± 0.01	0.75 ± 0.01
T/η	0.3 ± 0.01	0.33 ± 0.01	0.36 ± .01	0.36 ± .01
	Fluid Component			
$2T_{\|\|}'$	40.6 ± 0.5	40.0 ± 0.2	38.9 ± 0.5	39.1 ± 0.1
$2T_{\perp}'$	20.5 ± 0.1	21.1 ± 0.2	21.1 ± 0.2	21.4 ± 0.3
ΔL	3.6 ± 0.1	3.4 ± 0.4	3.15 ± 0.15	3.13 ± 0.2
S	0.51 ± 0.01	0.47 ± 0.02	0.47 ± 0.02	0.47 ± 0.03
T/η	1.30 ± 0.05	1.62 ± 0.1	1.62 ± 0.1	1.68 ± 0.3

intense, and the intensity and splitting of the resolved outer wings (due primarily to restricted lipids) decreases slightly (Figure 4, middle panel). To quantify these effects of hexanol on the two motional populations of lipids in the composite spectra, we used spectral subtraction to separate the spectra into the two single components (Mahaney et al., 1992). As shown in Table 2, increasing hexanol decreases the fraction of restricted lipid (f_R). The largest reduction occurs in response to the addition of the initial 10 mM hexanol (−6.2%), with smaller additional reductions occurring at 20 mM and 30 mM hexanol (−9.3% and −10.3% total change, respectively). The values of f_R obtained for the 10, 20, and 30 mM hexanol-containing samples are similar to those obtained for the control samples without hexanol recorded at temperatures 2, 3.5, and 4 °C higher, respectively.

Table 2 also lists the spectral parameters obtained from the 14-SASL difference spectra. The individual spectral components were characterized by the outer splitting, $2T_{\|\|}'$, the inner splitting, $2T_{\perp}'$, and the half-width at half-height of the low-field peak, Δ_L. $2T_{\perp}'$ was not resolved in the spectra of the restricted component because these lie in the slow motional regime of conventional nitroxide EPR spectroscopy (cf. Marsh, 1981, 1982). Hexanol decreases $2T_{\|\|}'$ in both

spectral components and increases $2T_{\perp}'$ in the fluid component spectrum, consistent with an increase in lipid chain mobility in both populations, but the mobility increase is far greater for the fluid lipids (about 4%) than that observed for the restricted lipids (about 1.5%). The trends in the data for Δ_L with increasing hexanol concentration are in agreement with those for the spectral splitting data: Δ_L increases for the motionally-restricted spectra and decreases in the motionally-fluid spectra, consistent with an increased lipid mobility in the presence of hexanol. The values of Δ_L become less reliable at higher hexanol concentrations, primarily due to inaccuracies in the difference spectra arising from the generally poorer subtraction endpoints, and therefore they report only qualitative effects. In general, the spectral parameter data support the observation (mentioned above) that the effects of hexanol on the spectra of 14-SASL in SR membranes are similar to the effects of an increase in temperature (ΔT_{eff}) for each of the two resolved motional components.

The observed changes in 14-SASL splittings were quantified in terms of S, the *effective* lipid hydrocarbon chain order parameter (eq 6, Table 2). Hexanol decreases the order parameter of the restricted lipids slightly (2.6% by 30 mM), but the effects are much greater for the fluid lipids (7.8% by 30 mM), with all of hexanol's effect occurring upon the addition of the first 10 mM hexanol. The order parameters were used to calculate the effective lipid hydrocarbon chain fluidity (eq 7) of the two populations of lipids. Consistent with the decrease in 14-SASL order, 30 mM hexanol increases the effective fluidity of the restricted lipids by only 13%. A much larger fluidity increase (24%) is observed in the unrestricted lipids at a much lower hexanol concentration (10 mM).

It should be noted that the effects of hexanol at 7 °C on the properties of membrane lipids in SR membranes do not correlate as well with effects on the activity of Ca-ATPase as do the hexanol-induced changes in Ca-ATPase oligomeric state and rotational mobility. The data of Table 2 show there is a pronounced tendency for the effects of hexanol on SR membrane lipid properties to be near-maximal by the lowest concentration of hexanol used (10 mM), while the effects of hexanol on Ca-ATPase activity (Figure 1, Ca-ATPase oligomeric state (Figure 3), and Ca-ATPase rotational mobility (Table 1) continue to increase at hexanol concentrations up to 30 mM.

Effects of n-Hexanol at 7 °C on 5-SASL and 14-SASL in Liposomes of Extracted SR Lipids. In order to study the effects of *n*-hexanol on the lipids of the SR membrane, free from the perturbing influence of the SR membrane proteins, we determined the effects of *n*-hexanol on the EPR spectra of 5-SASL and 14-SASL in multilamellar liposomes of extracted SR lipids. 5-SASL is a probe of the acyl chain region adjacent to the phospholipid polar head groups, and 14-SASL probes the region near the center of the lipid bilayer (cf. Mahaney & Thomas, 1991). Analysis of EPR spectra of 5-SASL and 14-SASL in multilamellar liposomes of SR lipids at 7 °C is shown in Table 3. The results are in qualitative agreement with the data obtain in SR membranes (Table 2) in that hexanol decreases the order parameter and increases the fluidity (T/η) of both 5-SASL and 14-SASL. The maximal effect, obtained at 40 mM hexanol, is to decrease the order parameters of 5-SASL and 14-SASL by 12.8 and 21.3%, respectively. Hexanol increases the fluidity parameter (T/η) of 5-SASL and 14-SASL by 66.5 and 71.9%, respectively. The effects of *n*-hexanol on both fluidity and order parameters are greater near the center of the phospholipid bilayer than near the phospholipid head groups. It is noteworthy that the effects of hexanol at 7 °C on the EPR-derived parameters of both 5-SASL and 14-SASL in multilamellar liposomes of SR lipids continue to increase at hexanol concentrations up to 40 mM. This is in contrast to the parameters of 14-SASL in intact SR membranes, described above, for which the effects of hexanol are near maximal at 10 mM hexanol.

Table 3: Influence of *n*-Hexanol on EPR Spectral Parameters and Derived Quantities of 5-SASL and 14-SASL in Protein-Free Multilamellar Liposomes of SR Lipids at 7 °C

spin label	[hexanol] (mM)	$2T_{\parallel}'$	$2T_{\perp}'$	S	T/η
5-SASL	0	57.2	16.6	0.7284	0.397
	10	55.8	16.96	0.6991	0.466
	20	55.05	17.34	0.6768	0.527
	30	54.83	17.53	0.6675	0.555
	40	53.67	18.05	0.6354	0.661
14-SASL	0		21.21	0.4619	1.71
	10		21.62	0.4363	1.97
	20		22.13	0.4044	2.35
	30		22.44	0.3850	2.61
	40		22.79	0.3631	2.94

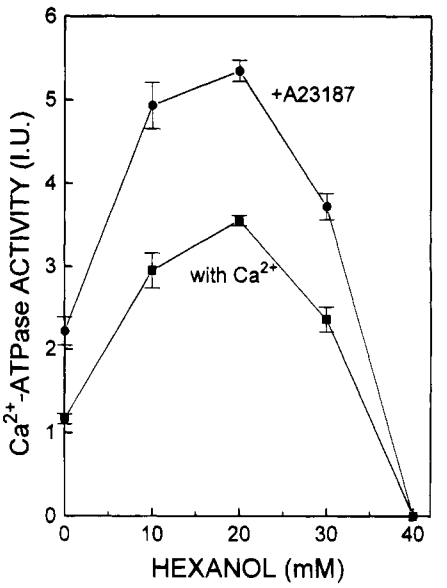

FIGURE 5: The influence of *n*-hexanol on the Ca-ATPase activity of skeletal SR determined as described in the Materials and Methods at 25 °C and pH 7.0 in the presence of 0.2 μg/mL of A23187 (●) or in its absence (■). SR (20 μg protein) was assayed in a 1 mL volume containing 100 mM KCl, 100 mM triethanolamine, 5 mM $MgCl_2$, 3 mM Na_2ATP, 0.5 mM EGTA, 0.55 mM $CaCl_2$, 1.5 mM PEP, 0.2 mM NADH, 15 units/mL of pyruvate kinase, and 30 units/mL of lactate dehydrogenase.

Effects of n-Hexanol at 25 °C

Effects of n-Hexanol on Ca-ATPase Activity at 25 °C. Hexanol at 25 °C has biphasic effects on Ca-ATPase activity (Figure 5). This is in marked contrast to the monotonic increase in enzyme activity with increasing hexanol concentration at 7 °C. At concentrations of 10 and 20 mM, there is a concentration-dependent increase in Ca-ATPase activity. At 30 mM hexanol the Ca-ATPase activity is less than at 10 and 20 mM hexanol, but still about 68% greater than in the absence of hexanol. At 40 mM hexanol there is

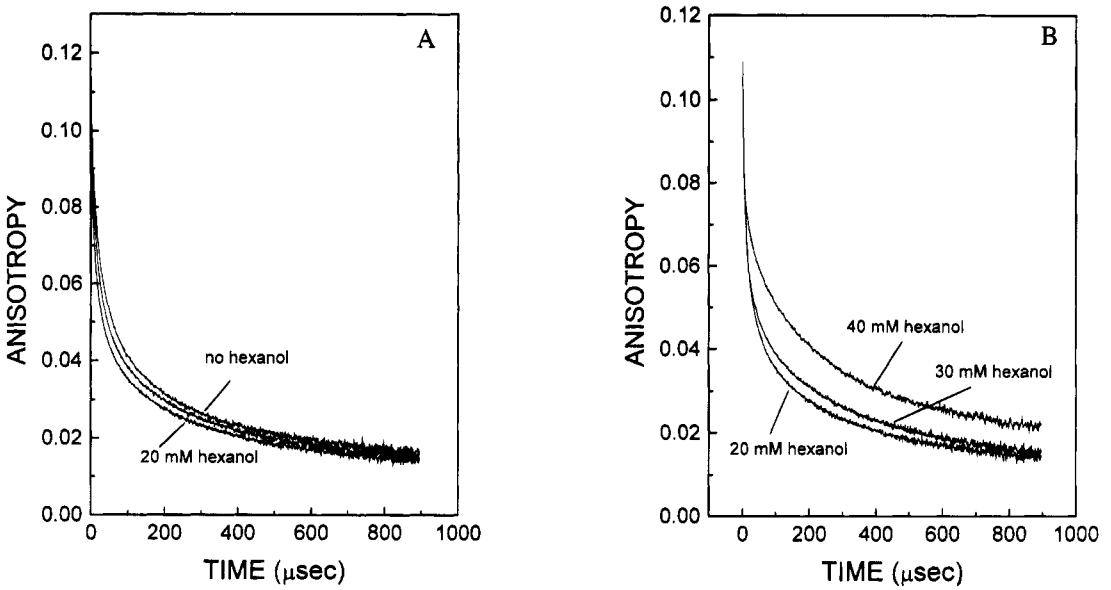

FIGURE 6: Time-resolved phosphorescence anisotropy of ERITC-SR in standard buffer at 25 °C (A) in the absence of *n*-hexanol and in the presence of 10 or 20 mM hexanol and (B) in the presence of 20, 30, or 40 mM *n*-hexanol. Each curve is the average of at least six separate data acquisition runs.

almost complete inhibition of Ca-ATPase activity. The inhibition of Ca-ATPase activity by 40 mM hexanol is largely reversible. When SR in 40 mM hexanol was diluted with buffer to 20 mM hexanol and incubated for 10 min at 25 °C, the Ca-ATPase recovered to (on the average) 90% of the Ca-ATPase activity of the SR at 20 mM hexanol (data not shown). This suggests that exposure to 40 mM hexanol did not denature the Ca-ATPase irreversibly under the conditions of our experiments.

These results are similar to those reported for hexanol in skeletal SR by Kondo and Kasai (1973), Hara and Kasai (1977), and Melgunov et al. (1987). The activity peak at about 20 mM is very similar to that observed by Hara and Kasai at 20 mM hexanol. In the earlier study (Kondo & Kasai, 1973) the peak activity was observed at about 15 mM hexanol. These previous studies were done at room temperature, while our experiments were carried out at 25 °C, which may account for the slight differences between the results. Kondo and Kasai (1973) found that the effects of a mildly inhibitory concentration of methanol could be reversed by diluting the incubation mixture to lower the methanol concentration, but that the effects of a concentration of methanol that caused almost complete inhibition of the Ca-ATPase could not be reversed. They attributed the inability to reverse the effects of high levels of methanol to irreversible denaturation. Our results suggest that under our conditions, the effects of 40 mM hexanol were largely reversible.

The observations of Karon and Thomas (1993) with halothane in skeletal SR have significant parallels to our findings with hexanol. At lower temperatures (4 and 7 °C) halothane caused a stimulation of Ca-ATPase activity that was monotonic with halothane concentration. At higher temperatures (15 and 25 °C), halothane had a biphasic effect, stimulating Ca-ATPase at lower levels and inhibiting at higher halothane concentrations.

Effects of n-Hexanol at 25 °C on Phosphorescence Anisotropy. Figure 6 shows the decay of the phosphorescence anisotropy of ERITC-labeled SR in the absence of *n*-hexanol and in the presence of 10, 20, 30, and 40 mM hexanol in the buffer solution. The presence of 10 or 20 mM *n*-hexanol (Figure 6A) increases the rate of decay of anisotropy in a concentration-dependent manner. Higher levels of *n*-hexanol (Figure 6B) reverse this effect. The anisotropy decay in the presence of 30 mM hexanol roughly resembles that in the absence of hexanol. In 40 mM hexanol the decay of anisotropy is dramatically slowed relative to the anisotropy decay in the zero hexanol control sample.

Table 4: Influence of *n*-Hexanol at 25 °C on Calculated Rotational Correlation Times, in μs ± Standard Error, Obtained by Fitting Eq 2 to the Data of Figure 6[a]

[hexanol] (mM)	ϕ_1	ϕ_2	ϕ_3
0	5.47 ± 0.22	33.0 ± 0.93	269 ± 9
10	5.26 ± 0.31	30.4 ± 1.18	243 ± 19
20	4.99 ± 0.21	30.0 ± 1.07	234 ± 11
30	4.44 ± 0.24	30.9 ± 1.71	301 ± 13
40	4.35 ± 0.31	30.9 ± 2.73	313 ± 19

[a] Shown are the average parameter values obtained by fitting equation (2) to at least 6 individual data acquisition runs.

Rotational Correlation Times. Fitting eq 2 with three components to the individual tracings that were averaged in Figure 6 gives the rotational correlation times shown in Table 4. The shortest rotational correlation time (ϕ_1) decreases monotonically with increasing hexanol concentration. ϕ_1 is decreased by 20.5% in the presence of 40 mM *n*-hexanol; the relative extent of the decrease is less than half that observed at 7 °C. The rotational correlation time of the intermediate component (ϕ_2) is barely affected by hexanol. The rotational correlation time of the largest rotating component (ϕ_3) is decreased by about 13% in the presence of 20 mM hexanol, but ϕ_3 *increases* at hexanol levels higher than 20 mM. At 40 mM hexanol ϕ_3 is about 16% higher than in the absence of hexanol. This contrasts with the effects of hexanol at 7 °C, where all three rotational correlation times were markedly decreased by hexanol in a concentration-dependent fashion. The biphasic changes in ϕ_3 suggest that the size of the average oligomer contributing to this rotational correlation time might increase at higher hexanol concentrations (see below).

Amplitudes. Fitting eq 2 to the individual tracings that were averaged in Figure 6 gives the amplitudes shown in

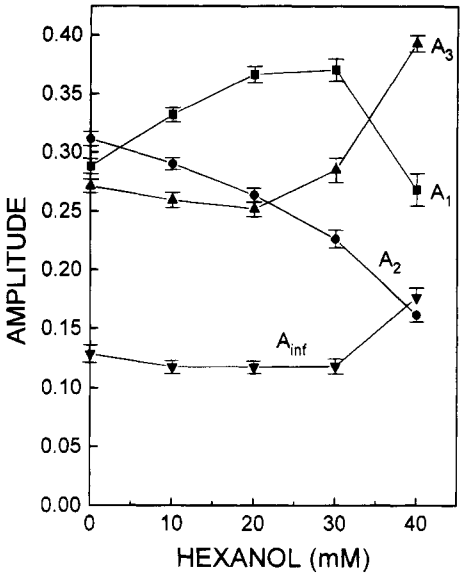

FIGURE 7: Influence of *n*-hexanol at 25 °C on the oligomeric state of Ca-ATPase. Normalized amplitudes (±standard error) result from fitting eq 2 to the data of Figure 6.

Figure 7. From zero to 20 mM hexanol, the mole fraction of the smallest component (A_1) increases with increasing hexanol, with concomitant decreases in the mole fractions of the intermediate (A_2) and the largest rotating components (A_3). At higher hexanol concentrations, 30 and 40 mM hexanol, there is a reversal of these trends with A_3 increasing dramatically at these alcohol concentrations. Between 30 and 40 mM hexanol, A_∞ increases significantly. The effects of hexanol at 25 °C at concentrations *up to 20 mM* are reminiscent of the effects of hexanol we observed at 7 °C, that is, an apparent decrease in the size of rotating oligomers of the Ca-ATPase. At hexanol concentrations *above 20 mM*, however, the opposite occurs: an increase in the apparent mole fraction of the largest rotating oligomers. At 40 mM hexanol the large increase in A_∞ suggests an increase in f_1, the mole fraction of oligomers that are too large to rotate appreciably on the time scale of the experiment. *The stimulation of Ca-ATPase activity by 10 and 20 mM hexanol correlates with an increase in the proportion of the Ca-ATPase present as the smallest rotating oligomers, while the inhibition of Ca-ATPase activity at 30 and 40 mM hexanol correlates with an increased proportion of larger oligomers of Ca-ATPase, with a diminished proportion of smaller oligomers. Our results suggest that the biphasic effects of hexanol at 25 °C on Ca-ATPase activity are due primarily to the biphasic effects of hexanol on the oligomeric state of Ca-ATPase.*

Effects of n-Hexanol at 25 °C on 5-SASL and 14-SASL in Liposomes of Extracted SR Lipids. Analysis of EPR spectra of 5-SASL and 14-SASL in protein-free multilamellar liposomes of SR lipids at 25 °C is shown in Table 5. The effects on the spin labels are qualitatively similar to those observed in SR lipids at 7 °C: hexanol causes a decrease in the order parameter (S) and an increase in the fluidity parameter (T/η) of both 5-SASL and 14-SASL. However, the effects of hexanol are significantly greater on 14-SASL than on 5-SASL, as was the case at 7 °C. *The effects of hexanol on the EPR parameters of 5- and 14-SASL in multilamellar liposomes of SR lipids depends monotonically on the hexanol concentration; there is no reflection of the biphasic behavior displayed in the effects of hexanol at 25 °C on the Ca-ATPase activity and on the time course of decay of phosphorescence anisotropy of ERITC-SR.*

Table 5: Influence of *n*-Hexanol on EPR Spectral Parameters and Derived Quantities of 5-SASL and 14-SASL in Multilamellar Liposomes of SR Lipids at 25 °C

spin label	[hexanol] (mM)	$2T_{\parallel}'$ (G)	$2T_{\perp}'$ (G)	S	T/η (K/cP)
5-SASL	0	51.44	18.66	0.586	0.87
	10	50.97	18.89	0.572	0.93
	20	50.84	19.14	0.563	0.99
	30	50.51	19.30	0.553	1.04
	40	50.05	19.70	0.534	1.15
14-SASL	0		23.32	0.330	3.53
	10		23.78	0.301	4.13
	20		24.27	0.271	4.89
	30		24.62	0.249	5.51
	40		24.95	0.228	6.17

The effect of general anesthetics, including the *n*-alcohols, to disorder and fluidize the phospholipids of biological membranes and of phospholipid bilayer preparations has been well documented (reviewed by Seeman (1972) and by Jain et al. (1978)). The fluidizing and disordering effects of *n*-alcohols on phospholipid bilayers of natural and model membranes has been demonstrated using steroid spin labels (Grisham & Barnett, 1973; Paterson et al., 1972), stearic acid spin labels (Lenaz et al., 1976), and the fluorescent probes perylene and 9-vinyl anthracene (Jacobson & Wobschall, 1974), 1-anilinonaphthalene 8-sulfonate (Lenaz et al., 1976), and (anthroyloxy)stearic acids (Zavoico et al., 1985). The *n*-alcohols from butanol to octanol were reported to increase the fluidity of microsomal membranes from chick embryo heart in direct proportion to their concentrations in the membrane (Zavoico & Kutchai, 1980).

Effects of Lidocaine at 25 °C

Effects of Lidocaine at 25 °C on Ca-ATPase Activity. Lidocaine inhibits the maximal activity of the Ca-ATPase; the decrease in maximal Ca-ATPase activity (in the presence of A23187) is roughly linear with increasing lidocaine concentration (Figure 8). The activity at 170 mM lidocaine is about 27% that in the absence of lidocaine. In the absence of A23187, low concentrations of lidocaine (below 35 mM) stimulate Ca-ATPase activity. The most straightforward interpretation of these results is that, in the absence of ionophore, lidocaine enhances the rate of efflux of Ca^{2+} from the SR (Johnson & Inesi, 1969; Boland et al., 1975) and in this way stimulates Ca-ATPase activity by relieving the inhibitory influence of Ca^{2+} accumulated in the SR lumen. At higher lidocaine concentrations (85 mM and above), the SR is apparently so permeable to Ca^{2+} that addition of A23187 only slightly stimulates Ca-ATPase. The effect of lidocaine on activity of Ca-ATPase in the presence of ionophore reveals the effect of the anesthetic on the Ca-ATPase itself, without the complications that result from changes in the permeability of the SR to Ca^{2+}.

Our results with lidocaine are consistent with previous studies of the effects of local anesthetics on Ca-ATPase of skeletal SR. Dibucaine, tetracaine, lidocaine, and procaine have been reported to inhibit the enzymatic activity of Ca-ATPase of skeletal SR (Boland et al., 1975; Suko et al., 1976). Local anesthetics also diminish Ca^{2+} uptake by skeletal SR (Johnson & Inesi, 1969; Wilcox & Fuchs, 1969;

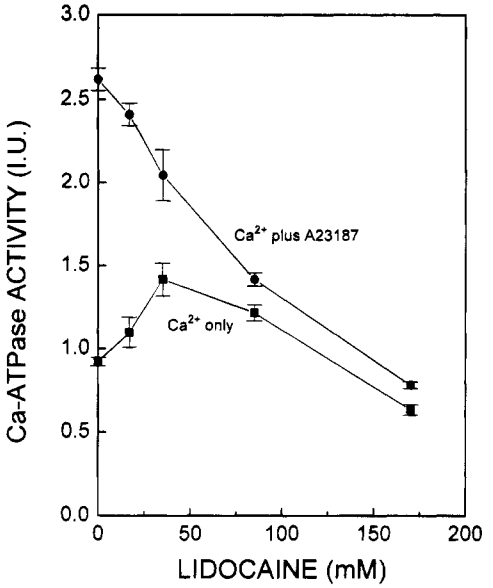

FIGURE 8: Influence of lidocaine on the Ca^{2+}-ATPase activity of skeletal SR determined as described in the Materials and Methods at 25 °C and pH 7.0 in the presence of 0.2 μg/mL of A23187 (●) or in its absence (■). SR (20 μg protein) was assayed in a 1 mL volume containing 60 mM KCl, 25 mM MOPS, 3.5 mM $MgCl_2$, 3.2 mM Na_2ATP, 0.25 mM EGTA, 0.3 mM $CaCl_2$, 1.6 mM PEP, 0.2 mM NADH, 10 units/mL of pyruvate kinase, and 10 units/mL of lactate dehydrogenase.

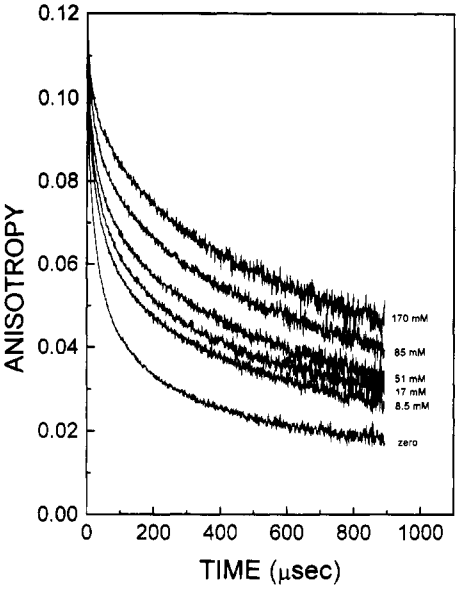

FIGURE 9: Time-resolved phosphorescence anisotropy of ERITC-SR in standard buffer at 25 °C in the absence of lidocaine and in the presence of various concentrations of lidocaine. Each curve is the average of at least 10 separate data acquisition runs.

Balzer, 1972; Boland et al., 1975). At low concentrations local anesthetics may decrease the passive efflux of Ca^{2+} from SR vesicles, but at higher levels local anesthetics appear to increase the passive permeability of skeletal SR to Ca^{2+} (Nash-Adler et al., 1980; Boland et al., 1976; Shoshan-Barmatz, 1988). The rate of formation of the phosphorylated intermediate of the Ca-ATPase from either ATP or from inorganic phosphate is markedly inhibited by local anesthetics (Suko et al., 1976). Local anesthetics may reduce the affinity of the Ca-ATPase for Ca^{2+} (Suko et al., 1976) and for ATP (Henao et al., 1991). Local anesthetics may also decrease the rate of hydrolysis of the phosphorylated intermediate (Henao et al., 1991).

Effects of Lidocaine at 25 °C on Phosphorescence Anisotropy. Figure 9 shows the effects of lidocaine on the phosphorescence anisotropy decay of ERITC-SR. *The effects of lidocaine are strikingly different from those of hexanol. The effect of lidocaine is to dramatically decrease the rate of decay of phosphorescence anisotropy.* Even the lowest concentration of lidocaine we used (8.5 mM), which had a small effect on Ca-ATPase activity, significantly slowed the rate of anisotropy decay. Lidocaine increases the anisotropies at long times. By contrast, hexanol (at least up to 30 mM) had no significant effect on the infinite-time anisotropy. The effect of lidocaine on the decay of anisotropy is dependent on the length of time the ERITC-SR has been preincubated in lidocaine prior to the TPA determination. The data shown in Figure 9 involved a 10 min preincubation. Time-dependence experiments suggest that the effect of lidocaine is relatively constant between 5 and 15 min of incubation. Before 5 min the effect is not fully developed. With longer incubation times, beyond 20 min, our results suggest that lidocaine causes aggregation of SR vesicles (as indicated by increased light scattering).

Rotational Correlation Times. Equation 2 was fitted to the data of Figure 9. The calculated rotational correlation times are shown in Table 6. The three rotational correlation times respond differently to the presence of lidocaine. ϕ_1 does not change significantly in response to lidocaine in the concentration range employed. ϕ_2 increases slightly with concentrations of lidocaine up to 85 mM; at 170 mM lidocaine there is a larger increase in ϕ_2. ϕ_3 increases monotonically with lidocaine concentration; ϕ_3 at 170 mM lidocaine is almost twice as great as in the absence of lidocaine.

Table 6: Influence of Lidocaine at 25 °C on Calculated Rotational Correlation Times, in μs ± Standard Error, Obtained by Fitting Eq 2 to the Data of Figure 9[a]

[lidocaine] (mM)	ϕ_1	ϕ_2	ϕ_3
none	5.80 ± 0.41	33.1 ± 1.37	273 ± 15
8.5	5.56 ± 0.33	39.4 ± 1.90	355 ± 20
17	5.84 ± 0.29	39.9 ± 1.61	397 ± 38
51	5.78 ± 0.69	42.3 ± 3.14	470 ± 42
85	5.73 ± 0.58	39.4 ± 2.65	467 ± 41
170	5.87 ± 0.89	51.5 ± 7.25	533 ± 46

[a] Shown are the average parameter values obtained by fitting equation (2) to at least 10 individual data acquisition runs.

Table 7: Influence of Lidocaine on EPR Spectral Parameters and Derived Quantities of 5-SASL and 14-SASL in Multilamellar Liposomes of SR Lipids at 25 °C

spin label	[lidocaine] (mM)	$2T_{\parallel}'$ (G)	$T_{\perp}'$ (G)	S	T/η (K/cP)
5-SASL	0	51.39	18.62	0.5868	0.863
	8.5	51.38	18.33	0.5963	0.819
	17	51.48	18.46	0.5933	0.833
	51	51.72	18.40	0.5985	0.810
	85	51.46	18.42	0.5944	0.828
	170	51.18	18.58	0.5853	0.870
14-SASL	0		23.24	0.3350	3.43
	8.5		23.29	0.3319	3.49
	17		23.40	0.3250	3.63
	51		23.53	0.3163	3.80
	85		23.58	0.3138	3.86
	170		23.70	0.3063	4.02

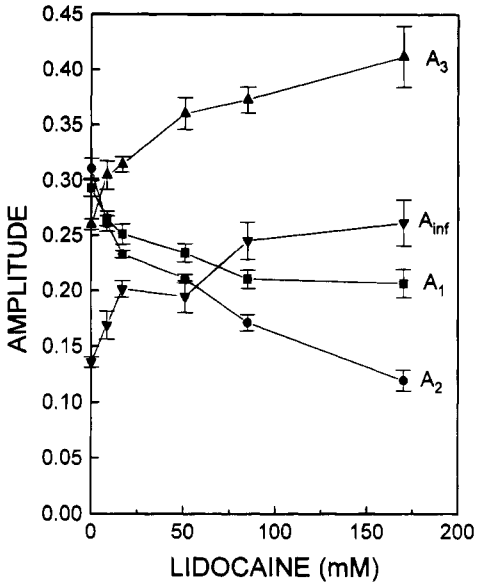

FIGURE 10: Influence of lidocaine at 25 °C on the oligomeric state of Ca-ATPase. Normalized amplitudes (±standard error) that result from fitting eq 2 to the data of Figure 9.

Amplitudes. The amplitudes of the three rotational components that were obtained by fitting eq 2 to the data of Figure 9 are shown in Figure 10. The effect of lidocaine is (a) to increase A_3, the mole fraction of the largest rotating component, (b) to increase A_∞, which partly reflects the mole fraction of aggregates that are too large to rotate on the time scale of the measurement, and (c) to decrease the mole fractions of the two smaller rotating species A_1 and A_2. The decrease in A_2 is striking. *Lidocaine causes smaller oligomers to form larger oligomers and aggregates of Ca-ATPase in the SR membrane; this correlates with the decreased maximal activity of Ca-ATPase in response to lidocaine.*

Effects of Lidocaine on 5-SASL and 14-SASL in Liposomes of SR Lipids at 25 °C. The effect of lidocaine on the EPR spectra of 5-SASL and 14-SASL in protein-free multilamellar liposomes of SR lipids are quite different from the effects of *n*-hexanol. Hexanol disordered and fluidized the phospholipid acyl chains to a greater extent for 14-SASL than for 5-SASL. *Lidocaine has effects on 5-SASL that are qualitatively opposite to its effects on 14-SASL.* Lidocaine (at least at concentrations up to 85 mM) increases the order parameter of 5-SASL and decreases its fluidity parameter. The effects of lidocaine on 14-SASL are to decrease the order parameter of 14-SASL and to increase its fluidity parameter. The effects of lidocaine on 14-SASL are in the same direction as those of hexanol, however, the effect of lidocaine on 14-SASL are much smaller than the effects we observed with hexanol: lidocaine at 170 mM caused an 8.6% decrease in S and a 17.2% increase in T/η of 14-SASL. *Lidocaine thus increases the order and decreases the fluidity of the acyl chains near the phospholipid head groups, but decreases the order and increases the fluidity of the acyl chains near the center of the bilayer.*

Our results are consistent with previous investigations of the effects of local anesthetics on membrane phospholipids. The influence of local anesthetics on membrane phospholipids has been characterized by NMR spectroscopy using phosphatidylcholines labeled with deuterium in the choline headgroup or on various carbons in the acyl chains (reviewed by Smith et al. (1991)). Local anesthetics were found to disorder the center of the lipid bilayer. The region of the acyl chain near the head group (i.e., the number 2 carbon of the acyl chain) showed little change in its order in the presence of local anesthetics. The head group itself may become more highly ordered by local anesthetics under certain conditions.

The effects of lidocaine on rotational correlation times of ERITC-SR can be tentatively interpreted in terms of lidocaine's effects on SR membrane lipids and on the oligomeric state of the Ca-ATPase. If we suppose that the smallest rotating oligomers do not change their average size in response to lidocaine treatment, then the constancy of ϕ_1 with increasing lidocaine suggests that the opposite effects of lidocaine on the order and motional properties of the center and headgroup regions of the acyl chain region of the membrane lipid bilayer result in no significant net effect on the rotational mobility of Ca-ATPase. Then the increases in ϕ_2 and ϕ_3 with lidocaine may be due to increases in the average size of Ca-ATPase oligomers contributing to these classes of rotating oligomers.

DISCUSSION

Overview. In this study we have characterized the effects of *n*-hexanol and lidocaine on the ATPase activity, oligomeric state, and rotational mobility of the Ca-ATPase in the SR membrane and on the properties of SR membrane lipids. *Our results suggest that a major mechanism by which hexanol and lidocaine influence the Ca-ATPase is by affecting the oligomeric state of the protein. Significant new findings are (1) that the biphasic effects of hexanol on Ca-ATPase activity correlate with biphasic effects of hexanol on the oligomeric state of the Ca-ATPase and (2) that the opposite effects of lidocaine and hexanol on Ca-ATPase activity correspond to opposite effects of these agents on the oligomeric state of the Ca-ATPase.*

Relation to Previous Studies. Previous reports from this laboratory have shown that ether (Bigelow & Thomas, 1987; Birmachu et al., 1990) and halothane (Karon & Thomas, 1993) influence the Ca-ATPase of skeletal SR and can affect the rotational mobility and the oligomeric state of the Ca-ATPase and the physical properties of the lipids of the SR membrane. In this study we find that *n*-hexanol, a general anesthetic, has effects on Ca-ATPase and on the properties of membrane lipids that are qualitatively similar to the effects of ether and halothane. By contrast, we find that the local anesthetic lidocaine has certain effects on the Ca-ATPase and on the lipids of the SR membrane that are different from the effects of the general anesthetics. Our results contribute to elucidating the molecular mechanisms which are responsible for the opposite effects of general and local anesthetics on the activity of the SR Ca-ATPase.

Previous studies from this laboratory support the interpretation that treatments that increase the rotational mobility of the Ca-ATPase or that promote dissociation of larger oligomers into smaller oligomers enhance the activity of the Ca-ATPase (Bigelow et al., 1986; Bigelow & Thomas, 1987; Squier & Thomas, 1988; Squier et al., 1988a; Birmachu & Thomas, 1990; Karon & Thomas, 1993; Voss et al., 1991, 1994). By contrast, treatments that promote formation of larger oligomers of Ca-ATPase appear to decrease the enzymatic and transport activity of the Ca-ATPase (Voss et al., 1991, 1994; Mahaney et al., 1992). Regulation of the

activity of Ca-ATPase by modulating its oligomeric state appears to be physiologically important in SR of cardiac muscle (Voss et al., 1994). Previous studies have also indicated that the activity of the Ca-ATPase can be influenced by the fluidity of SR membrane lipids (reviewed by Thomas and Mahaney (1993)) and by boundary lipid characteristics (Bigelow & Thomas, 1987).

Interpretation of Results of TPA Experiments. We have employed TPA measurements on ERITC-labeled SR to investigate the influences of hexanol and lidocaine on the oligomeric state of Ca-ATPase in the SR membrane and on the rotational rates of the various classes of oligomers. It is appropriate to emphasize the limitations of our interpretations of the TPA determinations.

The decision to analyze the TPA data in terms of three rotating components is an operational decision based on the finding that eq 2 and three exponential components fits the TPA decay curves better than two components, but that using four exponential components does not result in a significant improvement in the value of χ^2 for the fit. Birmachu et al. (1990) used their rotational correlation times in the Saffman–Delbrück equation (eq 5) to estimate the sizes of the three classes of oligomers of the Ca-ATPase. Their calculated effective radii are most consistent with the smallest rotating species being monomers, the intermediate-sized class being dimers, and the largest rotating oligomers being tetramers. These assignments should be regarded as tentative, but they provide a useful framework for interpreting the TPA results.

Our results suggest (Figure 3) that there is no significant change in A_∞ at any hexanol concentration at 7 °C. Equations 3 and 4 show that A_∞ includes contributions from the mole fraction of oligomers that are immobile (due, presumably to their large size) on the time scale of the experiment and from the orientation of the probe relative to the membrane normal. The simplest interpretation of the constancy of A_∞ with increasing hexanol is that neither the mole fraction of larger oligomers nor the angle of the probe relative to the membrane is changed by hexanol. It is possible, but we believe less likely, that there are offsetting changes in these two parameters as hexanol is increased. The data of Figure 3 indicate that there is a dramatic decrease in A_3, the apparent mole fraction of the largest oligomers, with no significant change in A_∞. The simplest interpretation of this finding is that there is no change in the orientation of the ERITC relative to the membrane normal as a result of the dissociation of the largest rotating oligomers into smaller species. This suggests that when larger oligomers dissociate, the orientation of the Ca-ATPase relative to the membrane does not change significantly.

Effects of n-Hexanol on the Ca-ATPase of SR. The observations of Karon and Thomas (1993) with halothane in skeletal SR have significant parallels to our findings with hexanol. At lower temperatures (4 and 7 °C) halothane caused a stimulation of Ca-ATPase activity that was monotonic with halothane concentration. At higher temperatures (15 and 25 °C), halothane had a biphasic effect, stimulating Ca-ATPase at lower levels and inhibiting at higher halothane concentrations.

The different responses to hexanol at 7 and 25 °C may be due to markedly different concentrations of hexanol in the SR membrane at the two different temperatures. Available data suggests that the solubility of hydrophobic and amphiphilic molecules in phospholipid bilayers increases significantly with increasing temperature. Katz and Diamond (1974) found that the partition coefficients of 16 nonelectrolytes between liposomes of dimyristoyl lecithin and water increased with increasing temperature. The solutes they studied included several aliphatic and aromatic alcohols. De Young and Dill (1988) found that the partitioning of benzene into multilamellar phospholipid vesicles increased with increasing temperature. They found a close positive correlation between the surface density of phospholipid molecules and the solubility of benzene in the bilayer membrane, independent of the manner in which the phospholipid surface density was altered (temperature, cholesterol, phospholipid acyl chain length). A plausible interpretation of our results is that at low intramembrane concentrations the predominant effect of hexanol is to promote the formation of smaller oligomers from larger ones, thereby stimulating the activity of Ca-ATPase, but at higher membrane concentrations hexanol enhances the formation of larger, less active oligomers of Ca-ATPase. At 7 °C and 30 mM hexanol it may be that the concentration of hexanol in the SR membrane remains in the stimulatory range, but that at 25 °C, due to the greater solubility of hexanol in the membrane at the higher temperature, 30 mM hexanol in the aqueous phase results in a concentration of hexanol in the membrane that begins to promote the formation of larger, less active oligomers of Ca-ATPase. Future research should address the issue of the molecular mechanism by which a low SR membrane hexanol concentration promotes formation of smaller oligomers of Ca-ATPase, while higher hexanol levels favor formation of larger oligomers.

Effects of n-Hexanol on the Rotational Correlation Times of ERITC-Labeled SR. Our data (Figure 1, Table 1) do not contradict the interpretation that enhanced rotational mobility *per se* of the Ca-ATPase enhances its enzymatic activity (Squier et al., 1988b; Birmachu & Thomas, 1990; Karon & Thomas, 1993; reviewed by Thomas & Mahaney (1993)). However, the close correlation of oligomeric state with Ca-ATPase activity in the presence of hexanol at 25 °C (Figures 5 and 7), in the absence of marked changes in rotational correlation times (Table 4), *favors the interpretation that the oligomeric state of Ca-ATPase is a stronger determinant of its enzymatic activity than the rotational mobilities of the different oligomeric species.* The data for lower concentrations of lidocaine (Figures 8 and 10, Table 6) are also consistent with this interpretation.

Effects of Hexanol on the Phospholipids of the SR Membrane. Lopes and Lauro (1991) used 14-PCSL (phosphatidylcholine spin-labeled at the 14 carbon of the *sn*-2 acyl chain) to study the effects of *n*-butanol, *n*-hexanol, and *n*-octanol at 2 °C on SR vesicles from rabbit white skeletal muscle. They concluded that all three alcohols, at concentrations that maximally activate Ca-ATPase, cause a decrease by as much as 30% in the fraction of phospholipids in the "boundary lipid" (those phospholipids whose motional properties are restricted by the presence of the protein). Our results at 7 °C (Table 2) using similar methodology to that employed by Lopes and Lauro with 14-SASL show effects of hexanol that are in the same direction as those observed by Lopes and Lauro, but our effects are much more subtle: 30 mM *n*-hexanol produced only a 10.9% decrease in the fraction of motionally-restricted lipids. Lopes and Lauro reported that 13 mM hexanol resulted in a 23% reduction in the restricted lipid component. The quantitative discrepancy

between our results and those of Lopes and Lauro may be due the 5 °C difference in temperature between the two studies.

Effects of Lidocaine on the Ca-ATPase. Even at the lowest concentration employed, 8.5 mM, lidocaine inhibited Ca-ATPase activity (Figure 8) and enhanced the formation of larger oligomers of Ca-ATPase (Figure 10). *These results lead us to propose a new hypothesis: that general anesthetics (at low concentrations) stimulate Ca-ATPase activity, while local anesthetics inhibit Ca-ATPase, because general and local anesthetics have opposite effects on the oligomeric state of the Ca-ATPase.*

Relationship of Concentrations Used to Anesthetic Levels of Hexanol and Lidocaine. The lowest hexanol concentration we employed is 10 mM. The data (Figures 1−3 and 5−7) suggest that we would have observed significant effects with 5 mM hexanol at both 7 and 25 °C. The level of hexanol required to abolish the righting reflex in 50% of tadpoles at room temperature is 0.7 mM (Pringle et al., 1981). The ED_{50} for block by hexanol of nerve conduction in rats at 37 °C has been reported to be 6 mM (Seeman, 1972). The levels of hexanol that decrease by 50% the Na^+ and K^+ currents of squid giant axons at 6 °C are, respectively, 3.5 and 9 mM (Haydon & Urban, 1986). The lowest level of lidocaine we used is 8.5 mM. Lidocaine is used clinically to produce local anesthesia at levels from 0.5 to 5% (Gilman et al., 1990), which corresponds to 18.5−185 mM. Thus, the lowest levels of hexanol and lidocaine used in this study are in ranges that may be relevant to their anesthetic action.

Relation to Possible Mechanisms of Anesthetic Action. It appears unlikely that Ca-ATPases are primary targets of local or general anesthetic action (Franks & Lieb, 1994). It is possible, however, that effects on Ca-ATPases, either those in plasma membranes (Kosk-Kosicka & Roszczynska, 1993) or in endoplasmic or sarcoplasmic reticulum, may play a role in modulating the primary effects of anesthetics on cells or in the mechanisms of the secondary effects of anesthesia. Moreover, an understanding of the ways in which anesthetics modulate the Ca-ATPase of SR might serve as a model for the mechanisms whereby anesthetics influence the proteins that are their primary sites of action.

Voltage-gated Na^+ channels in sensory neurons are frequently stated to be the primary site of action of local anesthetics. Local anesthetics, at clinically-relevant concentrations, block action potentials and voltage-gated Na^+ channels (Ritchie, 1986). A recent review of the mechanisms of general anesthesia (Franks & Lieb, 1994) supports the view that the most likely primary targets of general anesthetics are inhibitory synapses in the central nervous system. Particularly for the $GABA_A$ receptor, the postsynaptic receptor at the most numerous inhibitory synapses in the brain, there is much evidence that general anesthetics, at clinically-relevant concentrations, potentiate the postsynaptic response to a presynaptic action potential or to GABA agonists. A current view is that the most important mechanism by which general anesthetics potentiate GABA-ergic transmission is by binding to $GABA_A$ receptors and enhancing the mean open time of the ion channel in response to a channel agonist (reviewed by Franks and Lieb (1994)).

Our results are consistent with the view that anesthetics alter the behavior of ion channels by affecting the interactions between their subunits or their transmembrane helices. Some ion channels are known to be multimeric (Caterall, 1988; Nicoll et al., 1990), such as the $GABA_A$ receptor (Schofield et al., 1987), the nicotinic acetylcholine receptor (Kistler et al., 1982), the voltage-gated K^+ channels (Jan & Jan, 1989), and several others. In other ion channels, such as voltage-gated Na^+ and Ca^{2+} channels, the active ion channel is formed by the association in the membrane of transmembrane helices from each of four repeating domains, with each domain having six transmembrane helices (reviewed by Caterall (1988)). The available data suggest that gating of both ligand-gated and voltage-gated ion channels involves sometimes subtle relative movements of channel subunits, of transmembrane helices that comprise the ion channel, or of those parts of the protein involved in gating the channel (Unwin & Zampighi, 1980; Caterall, 1988).

Our results show that anesthetics, both general and local, interact with the SR membrane in such a way as to modulate the interactions between Ca-ATPase monomers that determine the predominant oligomeric states of the protein. For this reason, the effects of anesthetics on protein−protein interactions in the SR membrane might be considered to be a model of the effects of anesthetics on the interactions between subunits of the $GABA_A$ receptor or on the interactions between different domains of the voltage-gated Na^+ channel. The opinion that appears to predominate currently is that the primary targets of anesthetic action are sites on membrane proteins (Franks & Lieb, 1994) and that effects of anesthetics on membrane lipid structure and dynamics are of secondary importance. Our results do not inform the debate over protein vs. lipid sites of anesthetic action. The changes in the oligomeric state of the Ca-ATPase that we observe in response to general and local anesthetics might be due to the anesthetics influencing interactions between Ca-ATPase monomers or to anesthetic effects on interactions between Ca-ATPase and the surrounding membrane lipids, or to effects on protein−protein *and* on protein−lipid interactions. It remains for further studies to determine the mechanisms by which general and local anesthetics influence the oligomeric state of Ca-ATPase and to pinpoint the sites where the anesthetics act to produce these effects.

Conclusions. In this study we have characterized the effects of *n*-hexanol and lidocaine on the enzymatic activity, the oligomeric state, and the rotational mobility of the Ca-ATPase in the SR membrane and on the properties of SR membrane lipids. *On the basis of our results we propose that a major mechanism by which anesthetics influence the Ca-ATPase is by affecting the oligomeric state of the protein. Two incompletely-understood aspects of anesthetic action on the Ca-ATPase that can be rationalized in terms of effects of anesthetics on the oligomeric state of Ca-ATPase are (1) the observation that general anesthetics at lower concentrations stimulate Ca-ATPase activity, while higher concentrations of general anesthetics may be inhibitory, and (2) the finding that local anesthetics inhibit the activity of Ca-ATPase, while general anesthetics (at lower concentrations) stimulate Ca-ATPase activity.*

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