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# The effects of *n*-alkanols on the lipid/protein interface of $Ca^{2+}$-ATPase of sarcoplasmic reticulum vesicles

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**The effects of ethanol, *n*-butanol, *n*-hexanol and *n*-octanol on lipid–protein interactions in sarcoplasmic reticulum vesicles (SRV) are investigated using the C-14 nitroxide spin-labeled phosphatidylcholine. *n*-Alkanols, which activate the $Ca^{2+}$-dependent ATPase of sarcoplasmic reticulum but decrease net $Ca^{2+}$ uptake by the vesicles, are shown to affect the lipids interacting with the protein surface. Spectral analysis revealed that increasing concentrations of the alcohols progressively displace and mobilize lipids from the lipid/protein interface. For butanol, hexanol and octanol maximally activated SRV, 23 to 30% of the protein-interacting lipids are displaced. Thus, the displacement of more than 30% of the annular lipids by these alkanols cause inhibition of the enzyme. The motional properties of the labels that remain restricted by the protein surface are unaffected by the alcohols. The degree of mobilization attained by the labels displaced from the interface is much greater than that observed in alcohol-treated dispersions of extracted lipids. We propose that the alcohol molecules interfere with the protein–lipid interactions creating fluid clusters around the proteins. These fluidized regions would affect the enzyme conformation, perturbing its function. Fluidized annular lipids apparently increase the number of ion-conducting defects around the enzyme, increasing $Ca^{2+}$ efflux, and thereby reducing net uptake.**

## Introduction

A wide variety of chemical substances that perturb the lipid moiety of the membranes can affect the function of several integral membrane proteins. Anesthetic agents are among these substances and their molecular interactions with the various components of the biological membranes can help to elucidate their mechanisms of action. The *n*-alkanols share the property of general anesthesia. At concentrations about one order of magnitude higher than those required for general anesthesia they also affect the function of ion channels such as the nicotinic acetylcholine receptor [1] and the $Ca^{2+}$-ATPase of sarcoplasmic reticulum vesicles [2,3]. During the forward catalytic cycle of this ATPase calcium is pumped into and accumulated by the vesicles. The *n*-alkanols cause an enhancement of the ATPase activity and a decrease of net calcium uptake. The alcohol concentration producing the maximum activity, $c_{max}$, depends on the number of carbon atoms of the alcohol chain. At concentrations higher than $c_{max}$ the activity decreases sharply. The good correlation between the values of $c_{max}$ [2] and the alkanol lipid/buffer partition coefficients [4] suggests that drug action is related to a hydrophobic site. A review on anesthetic sites of action is presented in Ref. 5. The modification of the lipid/protein interface is a possible pathway for drug action. The hydrophobic part of the surface of an intrinsic protein interacts with the membrane lipids. The protein conformation is stabilised by the lipid–protein interactions. Changes of the lipid environment would produce conformational changes with functional consequences. This approach has been attracting attention [6–9], and experimental evidence has been presented [10,11].

Electron paramagnetic resonance of nitroxide spin labels has been used to probe the lipid–protein interactions in protein-containing membranes [12–14], including the sarcoplasmic reticulum membranes [13,15,16]. The hydrophobic portion of the protein surface restricts the motion of the interacting lipids. Spectral analysis of spin labeled lipids can be used to quantify the interacting lipids and the degree of motional restriction. This method can also be used to

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investigate the effect of drugs on the lipid–protein interactions. Diethyl ether has been shown to affect the lipids at the protein interface of $Ca^{2+}$-ATPase of sarcoplasmic reticulum [11]. Several general anesthetics, including *n*-alkanols, also affect the protein-interacting lipids in acetylcholine receptor-enriched membranes [10].

Here, we use the phosphatidylcholine spin labeled at the C-14 position to investigate the effects of *n*-alkanols on the lipid–protein interactions in sarcoplasmic reticulum vesicles, and correlate these effects with previously observed functional effects.

## Materials and Methods

Sarcoplasmic reticulum vesicles containing more than 80% of light vesicles were prepared from rabbit skeletal muscle [17,18] in L. de Meis laboratory (Biochemistry Department, Universidade Federal do Rio de Janeiro) and stored in liquid nitrogen. The phosphatidylcholine spin label 14-PCSL was synthesized in A. Watts laboratory (Biochemistry Department, University of Oxford) as described by Marsh and Watts [12].

Vesicles in a 50 mM Tris buffer (pH 7.0), 0.3 mM $MgCl_2$, were labeled at a label to protein ratio of 1:100 (w/w). Considering the ratio of total lipid/protein (w/w) in SRV equal to 0.57 [12] the upper limit for the label to lipid ratio is 1:57. A solution of spin label in ethanol (10 mg $ml^{-1}$) was injected into a dilute membrane suspension as described in Ref. 19. The vesicles were pelleted, washed, and then resuspended in 1 ml of buffer. Aliquots of ethanol, *n*-butanol, *n*-hexanol, and *n*-octanol were added to 1 ml samples (≈ 4 mg in protein) to give the aqueous final concentrations appearing in the text. For *n*-octanol the partition into the vesicles reduces the aqueous final concentration. This effect was taken into account using the value of 387 for the lipid/buffer partition coefficient [4], and estimating the volume of the lipid portion of the samples by their protein content assuming a lipid density of 1.0 g/ml and a lipid/protein ratio of 0.57 (w/w). The samples were vigorously mixed and incubated for 2 h at room temperature, and then transferred to 100 $\mu$l capillaries in which the membranes were repelleted.

EPR measurements were carried out at 2 °C on a Varian V-2500 spectrometer interfaced with an IBM PC-AT computer through an AD500 board for data acquisition and accumulation. A modulation amplitude of 0.1 mT and a microwave power of 10 mW were used. Software for spectral manipulations was implemented and used in the spectral analysis. In some cases the difference between the high- and low-field base lines indicated the presence of a very broad component due to some unincorporated spin label aggregates [12]. This component was subtracted out following the same procedure as Ref. 10, using the spectrum of pure 14-PCSL aggregates in buffer.

The effective order parameter $S^{eff}$ was calculated according to Ref. 20, using the expression

$$S^{eff} = (A_{max} - A_{min})/[A_{zz} - (A_{xx} + A_{yy})/2]$$

where $A_{zz}$, $A_{xx}$, and $A_{yy}$ were taken as 3.36, 0.63, and 0.58 mT, respectively [21].

## Results

### *Two component spectrum of 14-PCSL in sarcoplasmic reticulum vesicles*

Spin-labeled phosphatidylcholine in sarcoplasmic reticulum vesicles presents an EPR spectrum that allows to distinguish between bulk fluid labels and motionally restricted labels interacting directly with the protein surface. The bulk labels have the same motional properties as the labels in dispersions of extracted lipids. Motionally restricted lipids exchange with bulk lipids [13,22]. The resulting EPR spectrum is somewhat different from a pure two component spectrum to an extent that depends on the exchange rate. At low temperatures (0 to 10 °C) the exchange rate is slow enough on the EPR timescale so that the natural membrane spectra are often well fitted using the sum of two spectra obtained from dispersions of extracted lipids at different temperatures [14]. Spectral subtractions, as performed by many authors [10,11,23,24], can be used to obtain the proportions and the line shapes of the two spectral components.

Fig. 1 shows the spectrum of 14-PCSL in sarcoplasmic reticulum vesicles at 2 °C and its spectral analysis. The extracted-lipid spectrum at 2 °C was chosen as the more mobile bulk-lipid component which gave the best result according to criteria described in Ref. 24. Subtracting 68% of the normalized extracted-lipid spectrum at 2 °C (Fig. 1B) from the SRV spectrum (Fig. 1A), we obtained the best shape for the restricted component (Fig. 1C). The restricted motion lipids correspond, therefore, to 32% of the total lipids in the membrane. The model spectrum that matches the restricted component is that of extracted lipids at −15 °C, which appears superimposed to it in Fig. 1D. The effective order parameters corresponding to the two spectral components of 14-PCSL in SRV at 2 °C are 0.86 for the motionally restricted one, and 0.48 for the more mobile one.

### *Effect of the n-alkanols on the EPR spectrum of 14-PCSL in SRV*

The effect of *n*-alkanols, from methanol to heptanol, on the ATPase activity of $Ca^{2+}$-ATPase of SRV was measured by Kondo and Kasai [2]. The ATPase

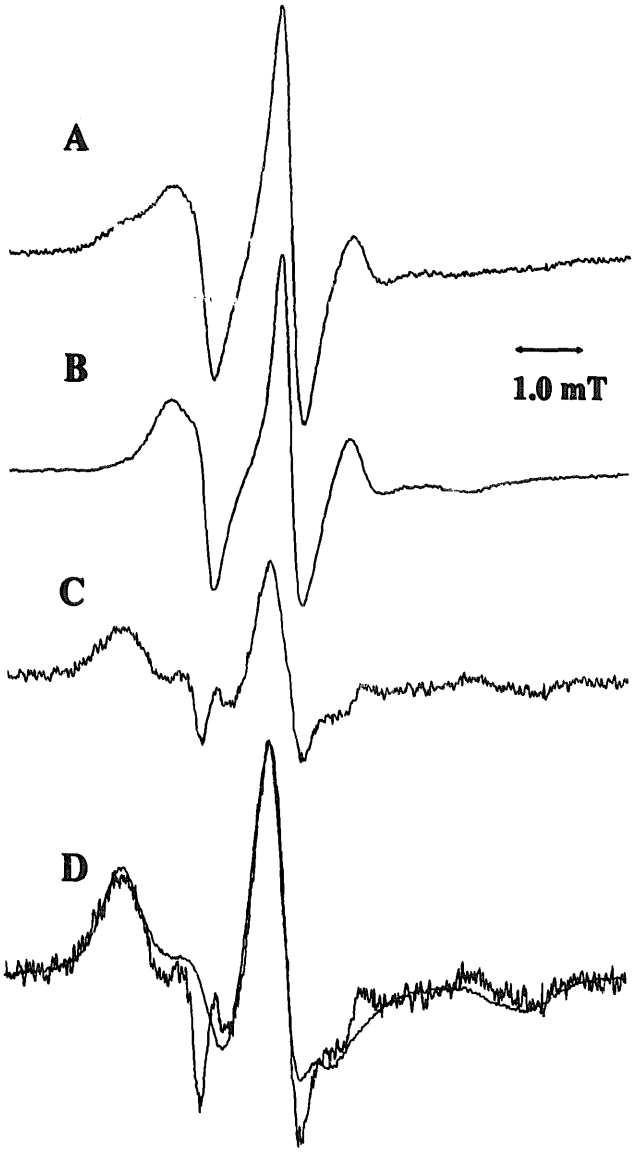


Fig. 1. EPR spectral analysis of 14-PCSL in sarcoplasmic reticulum vesicles. (A) Experimental EPR spectrum of 14-PCSL in SRV at 2 °C; (B) experimental EPR spectrum of 14-PCSL in vesicles of extracted lipids at 2 °C ($S^{eff} = 0.48$) (doubly integrated intensity is 68% of that in (A); (C) difference spectrum obtained by subtracting (B) from (A) (amplification factor 2) representing the restricted component; (D) experimental EPR spectrum of 14-PCSL in vesicles of extracted lipids at −15 °C (low-noise spectrum, $S^{eff} = 0.86$) superimposed to the difference spectrum enlarged 4-fold.

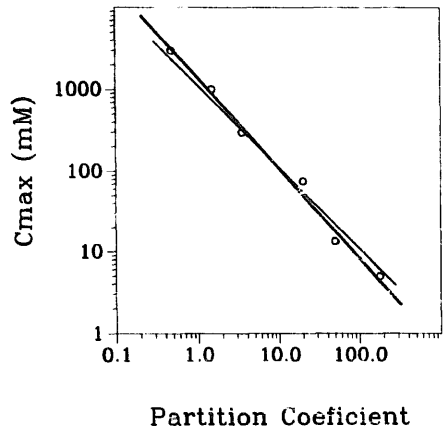


Fig. 2. The *n*-alcohol phospholipid/water partition coefficients, defined as the equilibrium solute concentration per unit volume in the lipid phase divided by the equilibrium solute concentration in the aqueous phase [4], vs. the *n*-alcohol concentrations at maximal ATPase activity [2]. From left to right, the points correspond to the *n*-alcohols from ethanol to *n*-heptanol. The thick line with a slope of −1.1 is the best fit for the data. The thin line is the best fit for a −1 slope line. Points on the thin line correspond to a 1 M intramembrane drug concentration.

activity of alcohol-treated vesicles first increases, reaches a maximum value, and then decreases with increasing alcohol concentration. It was found that the longer alcohol chains produce larger increments of ATPase activity. A plot of the *n*-alkanol concentration required to produce maximum ATPase activity, $c_{max}$, estimated from Fig. 1 in Ref. 2, against the lipid/water partition coefficient, $K_p$, for the *n*-alcohol [4] is shown in Fig. 2. The slope of the best fit line for these data is −1.1. This plot shows that the drug concentration in the lipid moiety required for maximal activation, $c_{max}$, is of the same order of magnitude for the *n*-alkanols tested. The best-fit line with a −1 slope corresponds to an intramembrane maximal activation concentration, $c_{membr}$, of 1 M. Since the slope is slightly less than −1.0, $c_{membr}$ decreases slightly with increasing chain length.

To correlate structural and motional effects of the *n*-alkanols with the observed functional effects we studied the EPR spectra of 14-PCSL in ethanol-, *n*-butanol-,

TABLE I

*Fraction of spin-labeled lipids mobilized and displaced from the protein lipid interface of SRV for various n-alkanols at the maximal activation concentrations, $c_{max}$; $c_{member}$ are the corresponding intramembrane concentrations, calculated as $K_p \cdot c_{max}$, where the values of the partition coefficients, $K_p$, were taken from Refs. 4 and 35; T is the temperature of the best-matching lipid spectra for the displaced lipids, and $S^{eff}$ is the order parameter, calculated as described in Materials and Methods.*

| Alcohol | $c_{max}$ (M) | $c_{membr}$ (M) | Displaced labels | $T$ (°C) | $S^{eff}$ |
|---|---|---|---|---|---|
| Ethanol | 3.0 | 1.4 | 10% | 19 | 0.35 |
| Butanol | 0.3 | 0.96 | 30% | 29 | 0.27 |
| Hexanol | $13 \cdot 10^{-3}$ | 0.65 | 23% | 23 | 0.31 |
| Octanol | $1 \cdot 10^{-3}$ | 0.39 | 28% | 27 | 0.29 |

*n*-hexanol-, and *n*-octanol-treated SRV, at a range of concentrations near the maximal activation concentrations, $c_{max}$. For octanol $c_{max}$ was extrapolated from a plot of $\log(c_{max})$ vs. the number of carbon atoms in the alkanol chain. The values for $c_{max}$ are presented in Table I.

The spectra in Figs. 3 A and B are representative of the effects of *n*-alkanols at $c_{max}$, and correspond to the control SRV and the *n*-butanol treated SRV samples, respectively. The apparent decrease in the spectral anisotropy reflects an increase in the mobility of a fraction of the spin labels.

To distinguish the effects on the interface from those on bulk lipids we first observed the spectral changes induced by the alcohols on dispersions of extracted lipids. Then, considering that the bulk lipids behave like the dispersions, we inferred the changes at the protein interface. The results on extracted lipids showed an increase in mobility on addition of alcohols. At 2 °C the mobilizations induced on the dispersions by alcohol treatments at $c_{max}$ were observed to be similar to that induced by a 6 °C increase in temperature ($S^{eff}$ varies from 0.48 to 0.46).

The analysis (Fig. 1) of the two component spectrum of 14-PCSL in SRV shows that there are 68% of spin labels away from the protein interface. Assuming that the changes on these labels are similar to those on extracted lipids, we subtract from the spectrum of alcohol-treated SRV the contribution due to bulk lipids (68% of an extracted lipid spectrum with the same mobility as the alcohol-treated one) to obtain the changes on the 32% spin labels originally localized at the protein interface. The results of this procedure for *n*-butanol at $c_{max}$ yielded the spectrum of Fig. 3D, which is the difference between the *n*-butanol treated spectrum in Fig. 3B and the extracted lipid spectrum at 8 °C in Fig. 3C (the higher temperature was used in order to allow for the decreasing molecular order induced by the alcohol on extracted lipids).

The spectrum in Fig. 3D showing the changes induced by *n*-butanol treatment on the 32% originally restricted spin labels is, in turn, a two-component spectrum. The positions of the extreme low- and high-field lines indicate that the motionally restricted component has the same parameters as that of unaffected SRV (Fig. 1C, $S^{eff} = 0.86$), but a lower intensity. Subtracting 70% of the motionally restricted component in Fig. 1C from Fig. 3D we obtain a mobile spectrum (Fig. 3E). Fig. 3F shows that this mobile component matches the extracted lipid spectrum at 29 °C ($S^{eff} = 0.27$). Thus, at $c_{max}$, *n*-butanol displaces and mobilizes 30% of the interfacial lipids.

Both the temperature of the pure lipid spectra, used to simulate the composite ones, and their effective order parameters are related to the mobility of the various membrane-lipid components. The spectral

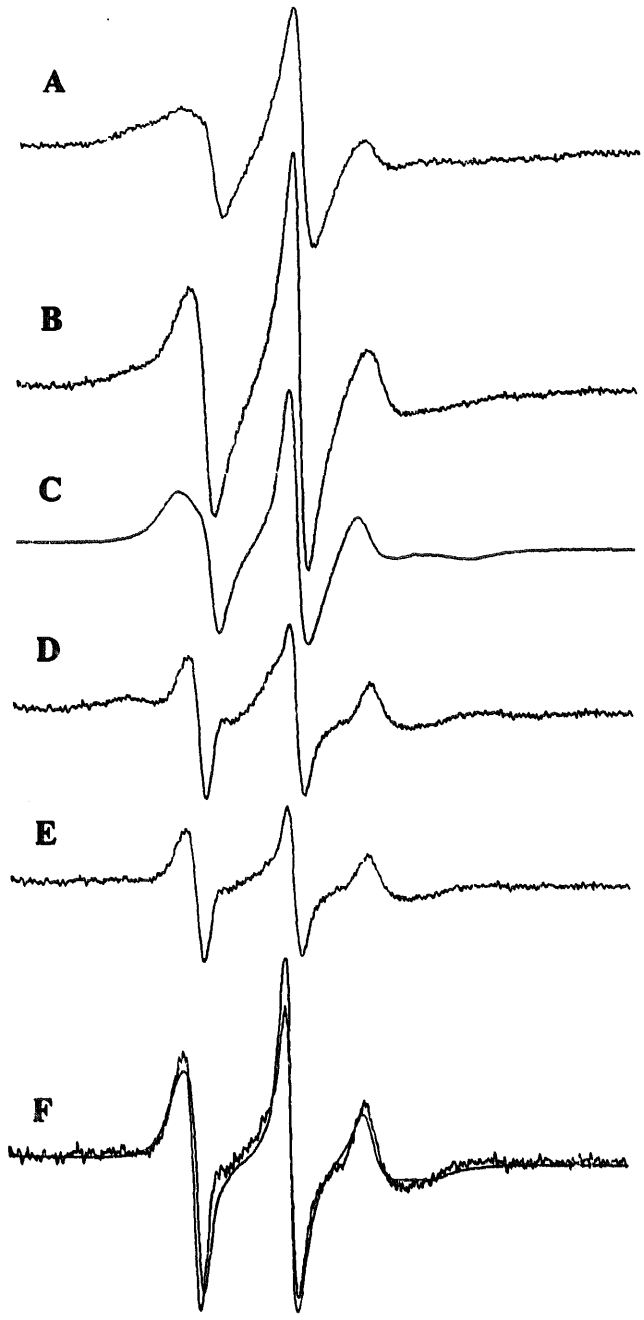


Fig. 3. The effects of the addition of *n*-butanol on the EPR spectum of 14-PCSL in sarcoplasmic reticulum vesicles. (A) Experimental EPR spectrum of 14-PCSL in SRV at 2 °C; (B) after treatment with *n*-butanol, 300 mM (doubly integrated intensity is the same as A); (C) Spectrum of extracted lipids at 8 °C. The intensity is 68% of spectrum A; (D) difference spectrum obtained by subtracting C from B. (E) difference spectrum obtained by subtracting from D, 70% of the restricted component in Fig. 1C; (F) Experimental spectrum of extracted lipids at 29 °C ($S^{eff} = 0.2$) superimposed to the difference spectrum E (amplification factor 2).

changes induced by *n*-butanol treatment at $c_{max}$, deduced from the analysis of the spectrum in Fig. 3 in terms of three components, are summarized below. The motional changes are quantified by $S^{eff}$ and by analogy with the temperature of the best-matching lipid spectrum. Extracted-lipids, thus, bulk lipids, are

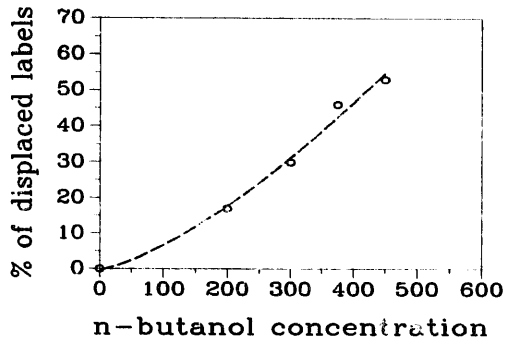


Fig. 4. Fraction of spin labeled lipids (14-PCSL) mobilized and displaced from the protein-lipid interface of SRV as a function of *n*-butanol concentration (mM) in the aqueous phase.

mobilized from 2 °C ($S^{eff} = 0.48$) to 8 °C ($S^{eff} = 0.46$), 70% of the interfacial lipids remain with the same mobility (−15 °C, $S^{eff} = 0.86$), and 30% of the interfacial lipids are mobilized from −15 °C to 29 °C ($S^{eff} = 0.27$). It is worth noting that after alcohol treatments some interfacial lipids become even more mobile than the alcohol-affected bulk lipids The results for the other alcohols are summarized in Table I. Except for ethanol, they are very similar.

Fig. 4 shows the fraction of lipids displaced from the interface for various *n*-butanol concentrations. The plot of the enzyme activity as a function of alcohol concentration in Ref. 2 showed a decrease in ATPase activity at concentrations greater than $c_{max}$, while the fraction of lipids displaced from the interface continues to increase.

Reversibility was tested with the hexanol treated samples. The samples were diluted in 1 ml buffer and pelleted by centrifugation after 2 h incubation. Up to the maximal activation concentrations the spectral changes caused by alcohol treatment are reversible. At higher concentrations reversibility is lost.

## Discussion

In this study we show that the enzymatic activation of $Ca^{2+}$-ATPase of SRV by the *n*-alkanols is accompanied by modifications of the molecular dynamics of the membrane lipids. The spectra of 14-PCSL in alcohol-treated SRV were deconvoluted by spectral subtractions and analysed in terms of three components: an alcohol-modified bulk lipid component, a motionally unaffected protein interacting component, and the most fluid component, which we attribute to labels mobilized and displaced from the protein surface. The assignment of the most fluid component to modified interfacial lipids is based on the observation that *n*-alkanols do not induce such an increase in fluidity of extracted lipids. Lipid bilayer membranes [25] and vesicles of lipids extracted from acetylcholine receptor-enriched membranes [26] also present small increase in fluidity under alkanol treatments. Thus, it seems likely that the most mobile component is related to the presence of membrane proteins.

Treatments with the alkanols decrease the fraction of protein-interacting motionally restricted spin labels. At maximal activation concentrations, the fraction of labels mobilized and displaced from the protein surface varies from 23 to 30% of the initial restricted population for butanol, hexanol and octanol, but is only is of the order of 10% for ethanol. There is no detectable change in the shape of the motionally restricted component. However, the fluidity of the displaced labels is much greater than that of bulk lipids. This suggests the uprising of some regions around the proteins, which are more fluid than the bulk-lipid environment itself. The increasing values of $c_{membr}$ in Table I, from ethanol to *n*-octanol, suggest that the longer alcohol chains are more efficient in disturbing the lipid/protein interface. Probably, bilayer-intercalated alkanols of increasing length are more effective in increasing the ratios of *gauche / trans* conformations of the methylene segments along the fatty acyl chains of the SR lipids near the molecularly rough protein surface. The results for *n*-hexanol are very similar to those obtained by Fraser et al. [10] in acetylcholine receptor-enriched membranes.

The intramembrane alkanol concentrations which produce the functional and dynamic changes described above are indeed higher than the concentrations which induce anesthesia (Table I). There is a possibility that the decrease of the restricted component is due to a statistical redistribution of labels between the interface and the bulk, because of the inclusion of alcohol molecules in the bilayer. To analyse this possibility we estimate the intramembrane fraction of alcohol to lipid molecules and the fractional increase in bilayer surface. Assuming a lipid density of 1 g/ml and an average lipid molecular weight of 780, the concentration of lipids in the bilayer is about 1.3 M. Using the values of $c_{membr}$ in Table I, we obtain alcohol to lipid molar ratios of 1.1 for ethanol, 0.74 for *n*-butanol, 0.5 for *n*-hexanol, and 0.3 for *n*-octanol. The average area of a phospholipid in the membrane is about 60 Å$^2$ [27,28], and the average area of a stearic acid in the bilayer was estimated as 20 Å$^2$ [29]. The area spanned by an alcohol molecule is probably smaller than the latter one, but we take it as an upper limit, which is more realistic for the longer alkanols. In this approximation, the fractional area spanned by alcohol molecules is three times less than the corresponding alcohol to lipid molar ratio. Hence, the increase of the membrane bilayer surface is 36% for ethanol, 25% for butanol, 17% for *n*-hexanol, and 10% for octanol. Comparing these numbers with the fractions of displaced labels (Table I), having in mind that the estimations above get worse for alkanols of decreasing length, we believe that

a statistical redistribution of labels does not totally account for the results. Furthermore, the lipids displaced from the protein surface are more mobile than the bulk lipids. This is incompatible with the statistical redistribution hypothesis.

The increased mobility of the displaced labels suggests that the alcohol molecules interacting with the protein surface create annular fluid clusters, which could be associated with the leak efflux of calcium that accompanies the increase of ATPase activity in alcohol-treated SRV [2]. Alcohols were shown to increase leakage of cations from lipid vesicles (for a review, see Ref. 5), probably by increasing the number of transient, ion-conducting defects normally present in the bilayer structure [30]. We suggest that alcohols preferentially create ion-conducting defects in the lipid/protein interface.

The spin-label results showed that the alcohol concentrations required to produce the maximum ATPase activity produce, except for ethanol, nearly the same mobilization and displacement of phospholipids from the lipid/protein interface. So, functional and interface-conformational effects are correlated.

Our results are different from those obtained with diethyl ether treated SRV. Using the 14-PESL, Bigelow and Thomas [11] found that diethyl ether, also a general anesthetic that activates the $Ca^{2+}$-ATPase, induces a decrease in viscosity of the restricted lipid population, corresponding to an increase in temperature from $-17$ to $-12\,^{\circ}C$. But they found no change in the proportion of the restricted component in maximally activated SRV. They concluded that ether mobilizes the lipid chains adjacent to the enzyme, although these chains remain much more restricted than the bulk lipid chains. Unlike the *n*-alkanols, ether activates the enzyme without increasing vesicle permeability for $Ca^{2+}$ ions [31,32]. The fact that ether and the *n*-alkanols act on interfacial lipids in different ways is consistent with their functional differences. It is also consistent with the hypothesis that the drugs which partition into the lipid moiety of biological membranes act on integral proteins through the lipid/protein interface.

The polar head-groups of the alcohols may play an important role in promoting conformational changes at the lipid/protein interface. Membrane-bound proteins owe their retention of conformation in the lipid bilayer to hydrophobic lipid–protein interactions, but it has been suggested that additional fixation is provided by lipid-protein hydrogen bonding [33,34]. Evidence has been presented for the activity and conformation dependence of membrane enzymes on the composition of the membrane hydrogen belts, i.e., the layers containing hydrogen bond acceptors and donors localized between the hydrophobic core and the polar zone facing the aqueous medium. It is worth noting that C=O oxygens of phospholipids are hydrogen-bond acceptors, which can form hydrogen bonds with the OH groups of the proteins. The OH groups of the alcohols can form hydrogen bonds with the C=O oxygens of the phospholipids and compete with the protein donors. Assuming that hydrogen bonding between phospholipids and protein donor groups helps to stabilize the proteins in the bilayer, the inclusion of *n*-alcohol head groups in the membrane hydrogen belts helps to weaken the phospholipid binding energy at the lipid/protein interface. This would explain in a molecular level the uprising of the fluid regions around the protein observed with the spin labeled phospholipids. The fact that ether does not have a hydrogen bond donor is consistent with these considerations.

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