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Interaction of alkanols and local anesthetics with spin-labeled Ca^{2+}-ATPase of sarcoplasmic reticulum vesicles

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Abstract

Alkanols and tertiary amine derivative local anesthetics modify the activity of Ca^{2+}-ATPase. In order to investigate the primary binding sites, associated to the functional changes, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase was labeled with maleimide derivative spin labels which bind covalently to SH groups of cysteine residues and allow to probe the regions of the protein close to those residues. The EPR measurements showed motional constraints induced by drug-treatment which indicate changes in the enzyme dynamics and structure. n-Alkanols are shown to affect some of the protein-bound labels by restricting their motion. There is, however, no correlation between the functional effects and the observed motional restriction, in the sense that concentrations of the different alcohols leading to the same functional effects do not induce the same degree of restriction. Dibucaine and tetracaine at functional relevant concentrations also restrict the movement of protein bound labels. But, in this case, correlation between spectral changes and functional effects is observed.

Keywords: ATPase, Ca^{2+}-; n-Alkanol; Dibucaine; Tetracaine; Spin label; EPR

1. Introduction

Anesthetics belong to diverse classes of chemical substances having in common the amphipathic character. This diversity puts an entangled problem on searching for the main targets of these substances and on sorting those which are important to the mechanism of anesthesia. It was pointed out that large anesthetic concentrations affect almost any target [1], and that it is important to consider only the effects at concentrations relevant to anesthesia. On the other hand, review papers [1,2] show how far we are from answering the question of what is the real target leading to anesthesia and how anesthetics work at the molecular level. With this in mind, clearly, the investigation of anesthetic interactions with well characterized biological membranes and their correlations with effects on membrane function helps to elucidate mechanisms of action, even if these effects occur at concentrations larger than those leading to anesthesia.

It is known [3] that the potency of an anesthetic increases roughly in proportion with its lipid/water partition coefficient, strongly suggesting an amphiphilic site for anesthetic molecules. The modification of the lipid/protein interface is a possible pathway for anesthetic action [4–9], in agreement with the above correlation. However, Franks and Lieb have claimed that increasing evidence has favoured the view that proteins and not lipids are the primary targets for anesthetics [1].

The n-alkanols share the property of general anesthesia. At concentrations about one order of magnitude higher than those required for general anesthesia they affect the function of ion channels, in particular the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) [10,11]. It was shown that the enhancement of the ATPase activity and decrease of net calcium uptake induced by the n-alkanols is accompanied by modifications of the molecular dynamics of the membrane lipids at the lipid/protein interface [12]. It was proposed that the alcohol molecules interfere with the protein–lipid interactions creating fluid clusters around the proteins, affecting the enzyme conformation, and perturbing its function [12].

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Local anesthetics such as dibucaine and tetracaine exert complex effects on the SR. These drugs are known to modify the rate of Ca^{2+} fluxes (either release or uptake) inducing blockage or enhancement depending upon drug concentration and experimental conditions [13–17]. These effects have been attributed to binding of local anesthetics to the phospholipids of the membrane [13,14], to changes in the protein/lipid interface [18], to blockage of Ca^{2+} channels [15,16] and to direct action on sites of the Ca^{2+}-ATPase [19]. Thus, the complex effects of local anesthetics on the functional behaviour of the SR suggest that these agents may have multiple sites of action. Thomas and Mahaney reviewed the influence of membrane properties on the activity of sarcoplasmic reticulum [20], and a single physical model was proposed to explain different functional effects of local and general anesthetics in SR: aggregation inhibits the Ca^{2+}-ATPase, and the oligomeric state of this protein is the strong determinant of its enzimatic activity [21,22].

Spin-labeling technique using nitroxide-labeled lipids is largely employed in membrane research, but the use of labels bound to integral membrane proteins is less common. Structural and dynamical effects of different drugs on proteins may be studied by using maleimide derivative nitroxide spin labels [23]. These labels bind covalently to SH groups present in cysteine residues, allowing to study the regions of the protein close to these groups. The Ca^{2+}-ATPase of SR contains 24 cysteine residues whose location was inferred from the analysis of the amino-acid sequence [24,25]. Among all these sulfhydryl groups there are only two very fast reacting groups and another two which belong to an intermediate class [26].

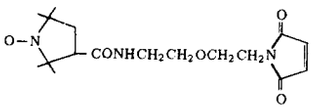
In this paper, we have used maleimide spin labels (MSL) to investigate the effects of n-alkanols and of the local anesthetics dibucaine and tetracaine on the protein portion of SRV, e.g., Ca^{2+}-ATPase, and we correlate these effects with previously observed functional effects.

2. Materials and methods

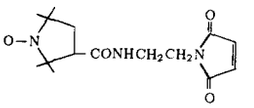
2.1. Materials

SRV from rabbit skeletal muscle prepared basically as previously described [27,28] were supplied by Dr. L. de Meis' laboratory (Biochemistry Department, Universidade Federal do Rio de Janeiro) and stored in liquid nitrogen. These samples are rich in Ca^{2+}-ATPase which constitutes approx. 90% of the protein content. n-Alkanols were purchased from Merck, local anesthetics (dibucaine and tetracaine hydrochloride) and the maleimide derivative spin labels 3-(2-[2-maleimidoethoxy]ethylcarbamoyl)-proxyl (MSL-4), 3-(2-maleimidoethylcarbamoyl)-proxyl (MSL-2) and 3-maleimido-proxyl (MSL-0) were purchased from Sigma. The molecular structure of these spin labels is given below.

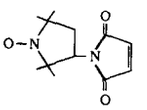
MSL-4



MSL-2



MSL-0



2.2. Spin labeling

Ca^{2+}-ATPase was labeled by incubation of SRV suspensions ($\approx$ 30 mg of protein per ml) with the desired spin label, at a label to protein ratio of 2–3, for about 24 h, at 8°C. Approximately 1 ml of the suspensions in Hepes 20 mM, at pH 7.4, with/without NaCl, was added to an appropriate aliquot of the stock solution of spin label in ethanol (10 mM), after evaporating the solvent. In order to eliminate the unreacted spin labels, the samples were centrifugated during 20 min at 17000 rpm and resuspended in the buffer solution at least twice. The EPR signal of the supernatant after the first washing showed that the quantity of unreacted spin label corresponded to less than 10% of the total quantity of spin label added. Unreacted label after the second washing was less than 1%.

2.3. Anesthetic treatment

The local anesthetics dibucaine and tetracaine were incorporated evaporating the solvent of an appropriate quantity of ethanol stock solutions of the drug ($\approx$ 10 mM) and adding, thereafter, 1 ml of the suspension of vesicles at a protein concentration of approx. 3 mg/ml. Aliquots of n-alkanols were added directly. Samples were vortex mixed during 1 min and incubated for 2 h. Aqueous local anesthetic concentrations were obtained spectrophotometrically after pelleting the SRV. For EPR measurements the pellets were redissolved in a small amout of buffer, transferred to capillaries and submitted to another centrifugation step. In the experiments to test if the anesthetics induce any radical reduction leading to variation of the total number of spins, concentrated suspensions of spin-labeled vesicles (50 mg protein per ml) were transferred directly to capillaries containing different amounts of dibucaine and butanol distributed over the internal surface of the capillaries. In this case the last centrifugation step was skipped.

Samples of labeled bovine serum albumin, from Sigma, and hemoglobin, freshly prepared, were used to test drug effects on water soluble proteins. In these cases, the excess of free label was eliminated by column filtration (Sephadex G-20, Sigma). 9 Lauryl ether, $C_{12}E_9$, used for SRV solubilization was purchased from Sigma.

2.4. EPR measurements and spectral analysis

EPR measurements were carried out at room temperature (22°C) on an X-band Varian E-9 EPR spectrometer interfaced with an IBM-PC for data acquisition and accumulation. A microwave power of 8 mW and modulation amplitude of 1 G (100 kHz) were used.

Software for spectral manipulations was implemented and used in the spectral analysis. All the EPR spectra were normalized to the same double integral after baseline correction based on the experimentally proven fact that the total number of spins does not change with drug treatment. The normalized spectra were deconvoluted by spectral substractions, and the fractions and line shapes of the spectral components were obtained as described in the next section.

3. Results

3.1. Spectral analysis of Maleimide Spin Labels in sarcoplasmic reticulum vesicles

Fig. 1 a, b, and c show the spectra of the three maleimide spin labels MSL-n (n = 4, 2, 0, respectively) in

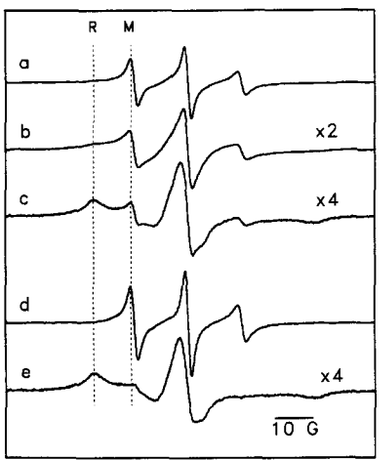


Fig. 1. EPR spectra of maleimide spin-labeled Ca^{2+}-TPase of SRV. (a): MSL-4, (b): MSL-2, (c): MSL-0, (d): difference spectrum obtained as $[(a)-0.375(c)]$ representing the mobile component and (e): difference spectrum obtained as $[(c)-0.06(d)]$ representing the restricted component. R and M indicate the low-field peaks of the restricted and mobile components, respectively. All the spectra are normalized to the same double integral with amplification factors indicated in the figure.

Table 1
Percentual contribution of the mobile component to the spectra in Fig. 1

	% fluid component
MSL-4	65
MSL-2	15
MSL-0	6

Ca^{2+}-ATPase of SRV. The spectrum of the shorter spin label MSL-0 and that of the longer one MSL-4 are basically due to spin labels in two different motional environments: one due to motionally restricted labels interacting with the protein side chains or grooves, and another due to mobile fluid labels, which, although bound to the protein, have their motion slightly affected by interactions with side chains. For MSL-0 the proportion of the motionally restricted is much higher than for MSL-4. The position of the restricted component low-field peak (R in Fig. 1) is the same for both MSL-0 and MSL-4, indicating that they have the same rotational correlation time. Then, the EPR profile of the mobile component can be obtained by subtracting from the MSL-4 spectrum (a) the fraction of spectrum (c) which cancels the low-field line R. The normalized resulting profile of this subtraction is shown in Fig. 1d. The EPR profile for the restricted component (Fig. 1e) was obtained subtracting 6% of the mobile component from the spectrum of MSL-0 (Fig. 1c).

The spectrum of the spin label MSL-2 (Fig. 1b), with intermediate length, has other motional components with intermediate values of rotational correlation times besides the two components obtained above. In this case, what we called the fraction of mobile component is the fraction of spectrum (d) necessary to cancel the peak M. The fraction of the mobile component present in each spectrum is given in Table 1.

All the spectral analyses performed to quantify the effects of anesthetics on the spin-labeled protein sites described below were based on previous normalization of all spectra. This is an adequate procedure if there is no radical reduction upon anesthetic treatment. If, in contrast, different motional environments have distinct levels of radical reduction the normalization of all spectra will lead to artifacts and misinterpretation. For this reason the presence of radical reduction upon anesthetic treatment was tested with the three maleimide spin labels. Butanol and dibucaine treatments at four of the highest concentrations were performed directly in the capillaries used for the EPR measurements and no centrifugation was performed after mixing. For the three spin labels the results showed that the double integrated intensity is the same as for untreated samples, within a standard deviation of 6%. Furthermore, the deviations from the mean have no correlation with the anesthetic concentration in the samples. In conclusion, there is no spin label reduction in our experiments, and normalization of spectra is a valid procedure.

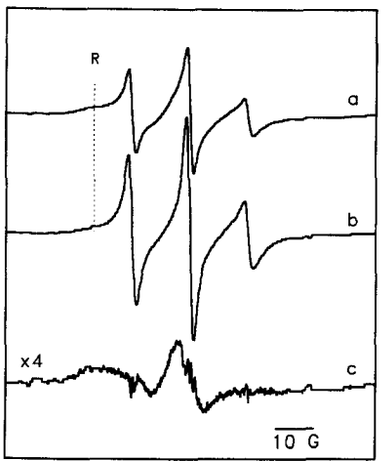


Fig. 2. Effect of the addition of n-butanol on the EPR spectrum of MSL-4-labeled Ca^{2+}-TPase of SRV and spectral analysis. SRV in Hepes 20 mM, KCl 100 mM at pH 7.4 and label to protein molar ratio 3:1. (a) EPR spectrum of MSL-4-labeled SRV treated with 400 mM butanol, (b) EPR spectrum of MSL-4-labeled SRV in buffer, normalized to the same double integral, (c) difference spectrum obtained as $[(a)-0.55(b)]$ (enlarged 4-fold) representing the restricted component. R indicates the low-field peak of the restricted component.

3.2. Effects of the n-alkanols on the EPR spectrum of MSL-n in SRV

n-Butanol treatment decreases the intensity of the mobile component of all the three maleimide spin labels in SRV and increases that of the restricted component. The spectrum in Fig. 2a is representative of this effect. The increase of the restricted component (R) shows that the drugs induce a strong restriction in the movement of a fraction of the mobile labels and not just a slight increase of the line widths. Actually, the increase of the restricted component is only observed with MSL-4 and MSL-2. The spreading of the restricted component spectrum over a much wider range of magnetic fields than the mobile one makes its signal to noise ratio much lower. For this reason the changes in the EPR spectra are quantified in terms of the population of mobile labels.

The EPR spectra were analyzed by the following spectral subtractions: different fractions f of the control spectrum (Fig. 2b) were subtracted from the spectra of n-butanol treated samples (e.g., Fig. 2a). The criterium used to obtain the fraction f for each alcohol concentration was elimination of the mobile component peaks M. The results are, therefore, restricted profiles like the one in Fig. 2c ($f = 0.55$ for the spectral subtraction in Fig. 2). Thus, in the presence of the alcohol, only a fraction f of the population of mobile labels remains unaltered, while $F = 1 - f$ of this population changes to a motionally restricted state. F is, therefore, the fraction of the mobile spin label population which is altered by drug treatment.

Fig. 3 shows the fraction F as a function of the concentration of n-butanol obtained from measurements

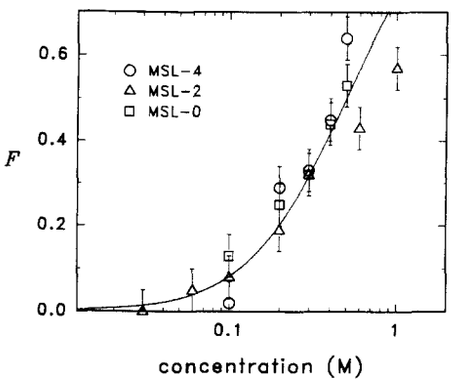


Fig. 3. Plot showing the dependence of the fraction F on the concentration of butanol. F is the fraction of the mobile component which becomes restricted after butanol treatment and it is obtained, as indicated in the text, from the spectral analysis of the EPR profiles of the Ca^{2+}-ATPase labeled with MSL. The solid curve is an aid to the eyes.

using the three different MSL. The results obtained with the different labels do not apparently provide any complementary information. These labels probably bind to the same sites in the protein, and the main differences among them are their fractions of mobile spectrum.

There is, however, a question to be answered: is the observed restriction effect of the n-alkanols correlated with their lipid/water partition coefficients? Since the functional effects correlate with the hydrophobicity measured by this coefficient, a positive answer to this question would favour the hypothesis of a direct action of alkanol molecules on Ca^{2+}-ATPase. To answer this question, experiments were performed with other n-alkanols, e.g., ethanol, n-hexanol, and n-octanol. In these cases the concentrations used were those inducing maximum ATPase activity, c_{max} [10], which are approximately proportional to the inverse of their lipid/water partition coefficients. The fraction F of mobile labels restricted by alcohol treatment at the concentration c_{max} for each n-alkanol is presented in Table 2, which also lists the lipid/water partition coefficients and the calculated intramembrane concentration. It is observed that the restriction effect decreases on going from ethanol to octanol. While ethanol restricts almost 50% of mobile labels, n-octanol has no effect. Moreover, despite n-octanol being more efficient than n-butanol in

Table 2
Concentrations of alkanols producing maximal ATPase activation (c_{max}), intramembrane concentrations (c_{mem}), calculated as $K_p \cdot c_{max}$, where the values of the partition coefficients, K_p, were taken from [29,30], and fraction (F) of mobile spin labels that become restricted after treatment with the n-alkanols at concentrations leading to the same functional effects

Alkanol	c_{max} (M)	c_{mem} (M)	F
Ethanol	3.0	1.4	0.47 ± 0.05
Butanol	0.3	0.96	0.31 ± 0.05
Hexanol	$17 \cdot 10^{-3}$	0.85	0.14 ± 0.05
Octanol	$1.6 \cdot 10^{-3}$	0.62	0.00 ± 0.05

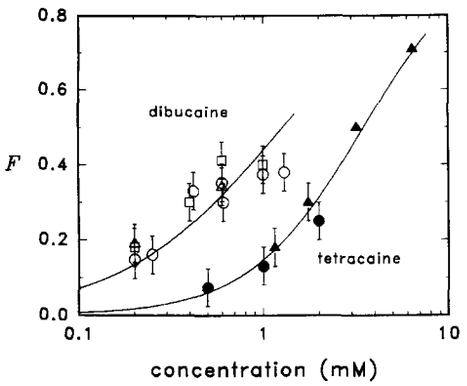


Fig. 4. The effects of dibucaine and tetracaine treatment on the EPR spectrum of MSL in sarcoplasmic reticulum vesicles. F is the fraction of the mobile component which becomes restricted after anesthetic treatment. The closed symbols represent tetracaine while the open ones are for dibucaine. Circles, triangles and squares are for MSL-4, MSL-2, and MSL-0, respectively. Solid curves are aids to the eyes.

perturbing the function of the enzyme, it is necessary a n-octanol membrane concentration 10-fold higher than that of n-butanol in order produce the same effect on the MSL spectra. In fact, $F = 0.31$ is obtained for n-butanol at c_{max} (0.3 M), which corresponds to ≈ 1 M in the membrane, while the same degree of immobilization is observed with 30 mM octanol, which corresponds to ≈ 11 M in the membrane. The answer to our question is, therefore, no. The n-alkanols restriction effects on Ca^{2+}-ATPase are not correlated with their lipid/water partition coefficients in the same way as their functional effects are.

3.3. Effect of dibucaine and tetracaine on the EPR spectrum of MSL-n in SRV

The effects of dibucaine and tetracaine on EPR spectra of maleimide spin-labeled SRV are similar to those observed for alkanol-treatment. Local anesthetics also induce changes in the sites to which the labels are bound such that the mobile labels become more restricted. The plot of the fraction F as a function of the anesthetic concentration (dibucaine and tetracaine) is shown in Fig. 4. The structural changes observed by EPR occur at concentrations at which these local anesthetics perturb the enzymatic function [17,31,32]. Contrary to the alkanols, the same degree of spin label restriction is observed for almost the same membranous concentrations. In fact, the effects of tetracaine and dibucaine are approximately equivalent for concentrations which are related by the same factor as the membrane/buffer partition coefficients for these drugs, e.g., the ratio of concentrations (tetracaine /dibucaine) giving place to spin label restriction such that $F = 0.3$ is in the range of 3 to 4, which coincides approximately with the ratio of partition coefficients estimated from the measurements of aqueous concentrations. This ratio is also close to that of concentrations producing similar functional disturbances on the enzyme [17,31,32]. This indicates a correlation between the effects on the protein and the changes induced in enzyme function.

Salt and pH effects were tested on dibucaine-treated SRV. Salt effects were not significant. The EPR spectrum of MSL-4 in control samples does not change for pH values above 6.4, while, for low pH (≈ 5.3), the fraction of the mobile component is much smaller and does not change upon dibucaine treatment. The restriction effect of dibucaine was detected for $6.4 < pH < 9.0$, suggesting that both the charged and neutral forms of the drug interact with the protein.

We also obtained spectra of spin-labeled SRV, solubilized with $C_{12}E_9$, as well as those of other soluble proteins, such as bovine serum albumin and hemoglobin, before and after dibucaine, and butanol treatment. In these cases, no relevant alterations were observed, even at concentrations much higher than those used to treat SRV samples. These negative results indicate that the effects observed in the case of the Ca^{2+}-ATPase are not a general property of proteins and that the action of these drugs may be associated to the presence of a lipidic environment.

4. Discussion

The interaction of anesthetics with sarcoplasmic reticulum vesicles is studied using maleimide-derivative spin labels with three different lengths. The spin labels covalently bind to the more reactive cysteines from Ca^{2+}-ATPase. The most probable localization for these cysteines is in the protein globular domains, where SH groups are abundant and more exposed to the aqueous phase than the alpha-helix domains which cross the membrane [24,25]. Since MSL-4 is sensitive to the lipid environment and to the oligomeric state of Ca^{2+}-ATPase [26] at least one of the labelling sites should be near the lipid protein interface. Labels at each binding site can have more than one motional environment, and the fraction of labels in each motional environment, as well as their respective correlation times, depend on the length of the label [33]. In Ca^{2+}-ATPase, about 35% of the longer one, MSL-4, still are in a strongly restricted motional environment (Table 1), and this fraction increases with decreasing label length. In the EPR time scale the severely restricted motional environment do not detect any further restriction.

Both classes of studied drugs, e.g. alkanols, which share properties of general anesthetics, and tertiary amine, local anesthetics, restrict the motion of maleimide spin labels bound to SR Ca^{2+}-ATPase, and this observed effect is quantitatively analyzed through the modifications induced in the spectral component of the most mobile labels. Spin label restriction was previously observed in red blood cells treated with anesthetics [34], and attributed to protein conformational changes. In Ca^{2+}-ATPase, the restriction effects are due to changes in regions of the protein adjacent to the more reactive cysteine residues. They could be

caused either by direct binding of the drug to protein sites near those residues, or by indirect effects due to propagated disturbance occurring on binding to other protein sites or to the lipidic matrix. The negative results we obtained with solubilized proteins treated with dibucaine indicate that the lipidic environment modulates the restriction. Drug induced aggregation of the Ca^{2+}-ATPase could be restricting MSL motion. In fact, Kutchai et al. reported the formation of large oligomers of Ca^{2+}-ATPase induced by local anesthetics, and a concentration dependent biphasic behaviour of dissociation or aggregation of small oligomers by hexanol [21].

Correlation of the restriction effect with functional effects was tested. Since correlation was found in the case of the tertiary amine local anesthetics, but not in the case of n-alkanols, we discuss them separately.

The good correlation between n-alkanol effects on ATPase activity and their lipid/buffer partition coefficients [10] suggests that drug action on the enzymatic function of SR Ca^{2+}-ATPase is related to a hydrophobic site. Recently developed studies on the action of n-alkanols at the lipid/protein interface of this same enzyme [12] using spin-labeled phosphatidylcholine (14-PCSL), showed that the n-alkanols displace and fluidize interfacial lipids creating annular fluid clusters, and that these changes correlate with the changes in activity and calcium permeability. Our results show that n-alkanols also disturb the protein inducing a motional restriction at sites near the most reactive cysteine residues. Nevertheless, the effects on the maleimide spin-labeled protein sites do not correlate with the functional effects. At the respective concentrations producing maximal activity [10,11], n-alkanols are far from producing the same degree of MSL restriction. The observation that the structural alteration detected by these protein-bound labels is not related to the alkanol induced functional changes indicates that the restriction of protein-bound spin labels is not a consequence of modulation of protein conformation by lipid–protein interactions. This conclusion favours the hypothesis that n-alkanols act on integral proteins through the lipid/protein interface but the presence of other function related protein site is not excluded. It also suggests a secondary site for alkanol binding at the protein, not related to the fluidization of the annular lipids. Hexanol was reported to dissociate or aggregate small oligomers of the Ca^{2+}-ATPase [21], depending on its concentration. We observed MSL restriction in the range of concentrations reported to induce dissociation. Thus, aggregation is probably not the cause of MSL restriction.

According to the results presented in Fig. 4, the local anesthetics dibucaine and tetracaine induce MSL restriction at functionally relevant concentrations. Moreover, equivalent degrees of restriction take place at dibucaine and tetracaine concentrations related by their lipid/buffer partition coefficients, obeying the rule: same intramembrane concentration, same effect. For tertiary amine local anesthetics there is also a good correlation between lipid/buffer partition coefficients and functional effects [17,31,32]. The observed restriction is, therefore, related to functional effects. The lipid/protein interface was tested for dibucaine binding using the phosphatidylcholine spin label 14-PCSL (unpublished result) but no changes were observed at concentrations affecting calcium uptake and ATPase activity. On the other hand, recent fluorescence measurements [35] showed that the highest affinity site for dibucaine in SRV is a lipid site near the membrane surface. Actually, a surface site for the local anesthetic explains why the label bound at the 14th carbon is not able to detect eventual changes at the lipid/protein interface. Some recent studies [17,19,36] suggest specific interactions of local anesthetics with membrane proteins, but in all cases protein/lipid interfacial sites are not excluded. The induced formation of Ca^{2+}-ATPase oligomers by local anesthetics, reported by [21], can account for the function related restriction effects reported here. The local anesthetic binding site that better accounts for the results obtained by the different authors is a protein site at the lipid/protein interface.

Despite the absence of information on the exact localization of the spin labels in Ca^{2+}-ATPase, pertinent conclusions are derived from our experiments. There are alkanol interacting sites in Ca^{2+}-ATPase not correlated with hydrophobicity or functional changes. In contrast, local anesthetics induce structural changes in the enzyme, which are coupled to functional disturbances. The most reasonable explanation is that these function related changes occur through local anesthetic binding to a superficial site at the lipid/protein interface. We propose that functional effects of both alkanols and local anesthetics are induced through the lipid/protein interface. The disturbance they produce in the lipid/protein interface is, however, different. Local anesthetics superficially bind to membrane lipids affecting protein domains, while alkanols deeply disturb the lipidic hydrophobic chains at the lipid/protein interface.

Acknowledgements

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