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LOCAL ANESTHETICS CAN INTERACT ELECTROSTATICALLY WITH MEMBRANE PROTEINS

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The fluorescent probe 1-anilinonaphthalene 8-sulfonate was used to examine the binding of spin-labeled local anesthetics to lipid model systems, to the membranes of human red blood cells, and rabbit sarcoplasmic reticulum. 1-Anilinonaphthalene 8-sulfonate exhibits two distinct fluorescent lifetimes when bound to these biological membranes. The shorter lifetime represents the probe associated with the purely lipid region while the longer lifetime is associated with the protein region. The spin-labeled local anesthetic quenches the fluorescence of both of these components as indicated by the decrease in the lifetimes. Since nitroxide free radicals are known to quench fluorophores upon 'contact', the results reflect the relative interaction of local anesthetics with membrane lipids and proteins. The evidence is consistent with the concept of multiple binding sites for local anesthetics in membranes. Local anesthetics, once intercalated into the bilayer, may diffuse laterally and interact with membrane components, lipid as well as proteins. In biological membranes, however, positively charged local anesthetics are better able to quench 1-anilinonaphthalene 8-sulfonate in protein regions, suggesting that the interaction between local anesthetics and membrane proteins can be electrostatic in nature.

While tertiary amine local anesthetics affect a variety of membrane-related functions including the blockage of sodium channels [1], the precise mechanism for any of these effects remains to be discovered. Earlier studies have shown that drug-lipid interactions correlate with anesthetic potency [2–5]. More recent results point to direct anesthetic-protein as well as anesthetic-lipid interactions [6–10]. In order to arrive at a molecular mechanism of anesthetic action, it is necessary to discover the forces of interaction between local anesthetics and the molecular components of the membrane.

Previous observations in this laboratory indicate that spin-labeled local anesthetics can quench the fluorescence of membrane-bound ANS in both the lipid and the protein regions of the erythrocyte membrane [11]. In the present communication, we have extended this investigation to model systems and other membranes. In particular, we have examined the relative involvement of electrostatic and hydrophobic interactions.

Materials and Methods

Chemicals

C6 and its spin-labeled analogs, C6SL, C2SL, and C6SLMeI (Fig. 1) were synthesized in our

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Abbreviations: ANS, 1-anilinonaphthalene 8-sulfonate; C2SL, 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl *p*-ethoxybenzoate; C6, 2-(*N*,*N*-diethylamino)ethyl *p*-hexyloxybenzoate; C6SLMeI, the methyl iodide salt of C6SL; C6SL, 2-[*N*-methyl-*N*-(2,2,6,6-tetramethylpiperidinooxyl)]ethyl *p*-hexyloxybenzoate; PC, phosphatidylcholine; PS, phosphatidylserine.

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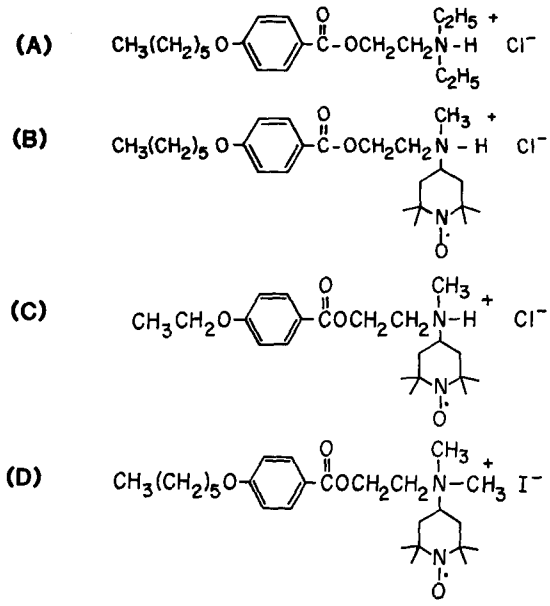

Fig. 1. (A) A local anesthetic, 2-(*N*,*N*-diethylamino)ethyl *p*-hexyloxybenzoate, abbreviated as C6, (B) Spin-labeled analog of C6 abbreviated as C6SL. (C) C2SL. (D) C6SLMeI, the quaternary analog of C6SL.

laboratory [12]. ANS was obtained from Polysciences in the form of ammonium salt and was recrystallized twice for fluorescence studies. Phosphatidylcholine (PC) and phosphatidylserine (PS) were obtained from Sigma; cholesterol was from Calbiochem. All other chemicals were reagent grade; chloroform and methanol were further distilled in our laboratory.

Membrane preparations

Phospholipid vesicles were prepared by a modified reverse phase evaporation method of Szoka and Papahajopoulos [13]. An aliquot of PC plus 20 mol% cholesterol in chloroform was evaporated to dryness. Then diethyl ether and 20 mM phosphate-buffered saline were added, and the mixture was agitated on a vortex mixer to form a one phase emulsion. Liposomes, formed by evaporation of the ether, were filtered through a 1.2 μm Millipore filter. Mixed PC/PS liposomes were made the same way except for the substitution of 20 mol% of phosphatidylserine for cholesterol.

Red blood cell membranes were prepared from either fresh human blood or outdated blood from the San Jose Red Cross by the method of Dodge et al. [14]. Whole cells were washed with 310 mosM phosphate buffer, pH 7.4, lysed in 20 mosM phosphate buffer, pH 7.4, and washed six-times. Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as the standard.

After excision, the white skeletal muscle from the hind legs of New Zealand rabbit was chilled on ice, trimmed of fat and connective tissue, and cut into small pieces. 100 g of tissue was added to 600 ml of buffer (100 mM KCl, 300 mM sucrose, 10 mM imidazole, pH 7.2, with 0.1 mM α-toluenesulfonyl fluoride) and homogenized in a VirTis 23, using two 300 s cycles at maximum speed. Cellular debris was removed by centrifugation at $1000 \times g$ for 10 min. The supernatant was filtered through four layers of cheesecloth presoaked with extraction buffer. Mitochondria were sedimented by successive centrifugations at 7000 and $10000 \times g$ for 15 min each. The resulting supernatant was then centrifuged for 1 h at 28000 $\times g$. The pellet was resuspended in sarcoplasmic reticulum storage buffer (100 mM KCl, 300 mM sucrose, and 10 mM imidazole, pH 7) at a protein concentration of 2–4 mg/ml.

Typically a sample consisted of 20 μl of membrane suspension in 3 ml of buffer. A small aliquot of concentrated ANS was added to give a final concentration of 6 to 10 μM ANS. For quenching studies, microliter quantities of the desired quencher, prepared in DMSO, were added to the membrane suspensions. Control experiments showed that this amount of DMSO has no effect on the lifetime measurements. The mixture was incubated at room temperature for 40 minutes before measurements were made.

Fluorescence measurements

Fluorescence lifetimes were measured with an Ortec 9200 Fluorescence Spectrometer. The sample was excited by a high pressure hydrogen flash lamp (EEY Scientific, La Jolla, CA) through a 380 nm three-cavity interference filter (Ditric Optics). The fluorescence is detected at 90 degrees from the excitation through a 450 nm cutoff filter (Optical Technology, Palo Alto, CA). At least 10^4 peak counts and approximately 10^6 total counts were taken for each fluorescence decay curve.

ANS is known to exhibit time-dependent spectral shifts [15] due to solvent reorientation about

the excited molecule during its fluorescence lifetime. In these quenching studies, since we are interested in the relative changes of the lifetimes rather than their absolute values, the use of a cutoff filter (instead of an interference filter) helps to average out the spectral shifts.

Data analysis

The observed fluorescence $F(t)$ is the convolution of the exciting lamp falsh $E(t)$ and the fluorescence response of the system to a delta function light flash $f(t)$:

$$F(t) = \int_0^t E(t-u)f(u)\mathrm{d}u = \int_0^t E(u)f(t-u)\mathrm{d}u$$

assuming that $f(u)$ has the form

$$f(u) = \sum_{i=1}^{n} a_i \exp(-u/\tau_i)$$

where a_i and τ_i are amplitudes and time constants, respectively, of the ith component.

The parameters a_i and τ_i are obtained by deconvolution using the method of moments [16]. In addition, moment index displacement [17] is incorporated in the procedure to automatically correct for scattered light, zero time shift, and slow lamp drifts.

The number of exponential terms used to fit the the fluorescence data is determined by the incrementation test [16]. By this test, the use of one additional exponential term than that presented in the results in this paper have led to either negative τ values or extremely small a values (by several orders of magnitude).

Results

Quenching of ANS in liposomes by C6SL

ANS readily binds to liposomes made of PC and cholesterol (4 : 1, mol/mol). The fluorescence decay of ANS in the presence of the liposomes is presented in Fig. 2A. The decay is a single exponential with a calculated lifetime of 7.6 ns. The local anesthetic C6SL quenches the fluorescence of

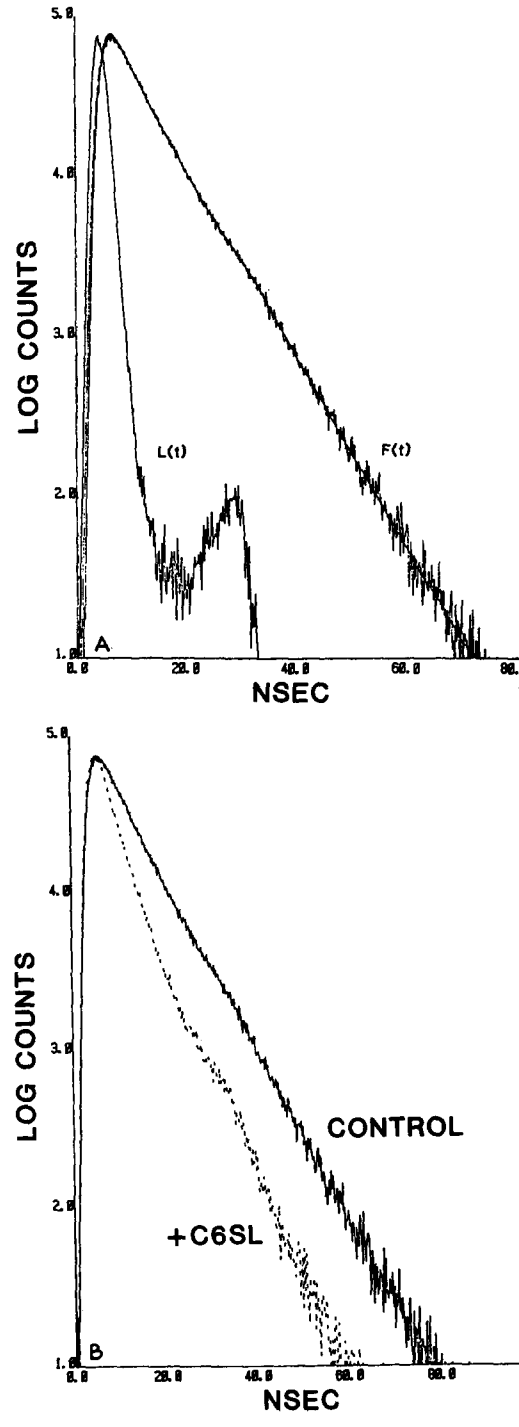

Fig. 2. (A) Nanosecond fluorescence decay curve of 10 μM ANS in the presence of PC/cholesterol liposomes. $L(t)$ is the lamp pulse and $F(t)$ is the recorded fluorescence. Superimposed on $F(t)$ is the theoretical fit of one exponential analyzed by method of moments. The lifetime is 7.6 ns. (B) Nanosecond fluorescence decay curves of 10 μM ANS labeled PC/cholesterol liposomes in the presence of and in the absence of 66 μM C6SL, pH 6.5.

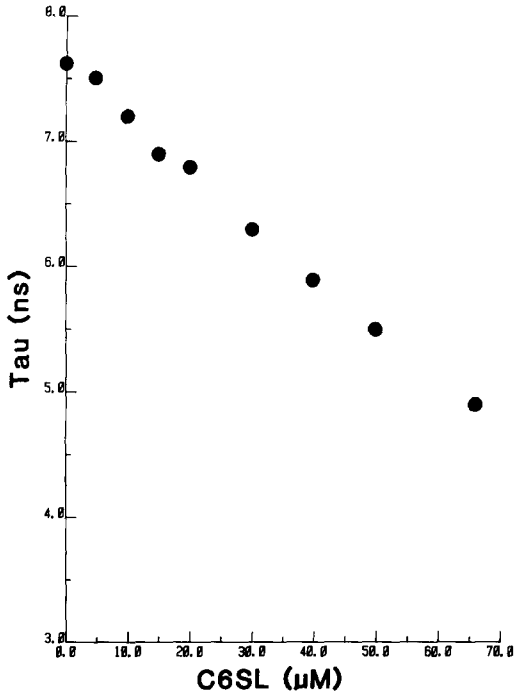

Fig. 3. The effect of the local anesthetic C6SL on ANS lifetimes in the presence of PC/cholesterol liposomes, pH 6.5.

liposome-bound ANS (Fig. 2B). At a concentration of 66 μM C6SL, the ANS lifetime is reduced from 7.6 ns to 4.9 ns, a 36% reduction. The amount of quenching is proportional to the concentration of C6SL (Fig. 3). Buffer pH in the range of 6 to 9.5 did not influence the lifetime of ANS with or without the presence of the quencher (Table IA).

TABLE I

ANS LIFETIMES

A	pH	ANS lifetimes in PC/cholesterol liposomes (ns)	
		liposomes	liposomes +66 μM C6SL
	6.5	7.6	4.9
	9.5	7.4	4.4
B	pH	ANS lifetimes in mixed PC/PS liposomes (ns)	
		Liposomes	Liposomes +66 μM C6SL
	7.0	7.5	4.8
	8.5	7.5	5.0
	9.7	7.4	4.8

Similar results were obtained when liposomes were made with PC and PS (4:1, mol/mol) (Table IB). The presence of PS in the membrane did not affect the fluorescence of ANS.

Binding of ANS to erythrocyte membranes

In erythrocyte membranes ANS fluorescence decay contains two distinct lifetimes (Fig. 4). The emission kinetics of membrane-bound ANS may be represented by the equation:

$$F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$$

where τ_1 is the shorter lifetime with a relative population of a_1, and τ_2 is the longer lifetime with a relative population of a_2. The absolute values of the a's are arbitrary, and depend on the number of counts in each recorded decay profile relative to the lamp pulse. The ratio a_2/a_1 is a more reliable parameter to monitor, representing the ratio of the

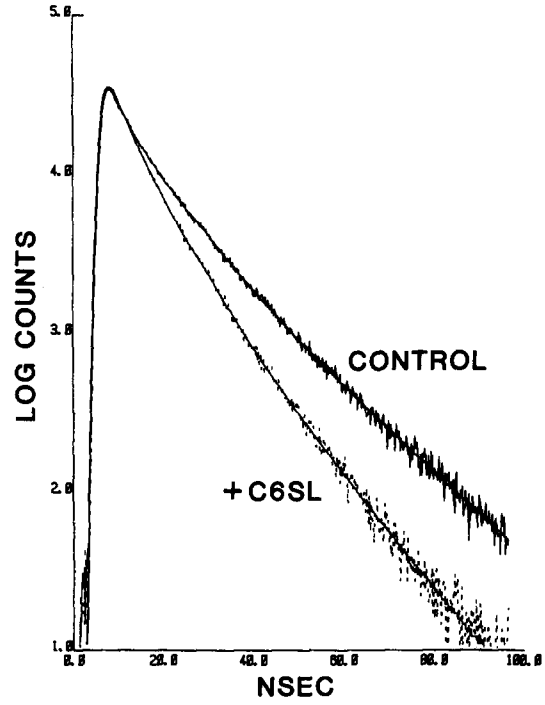

Fig. 4. The effect of 40 μM C6SL on the erythrocyte membrane-bound ANS fluorescence curve (dotted line). The solid line is the control fluorescence curve (erythrocyte ghost + ANS), pH 6. The smooth lines superimposed on the fluorescence curves are the corresponding theoretical curves based on the two exponential model (see text for details).

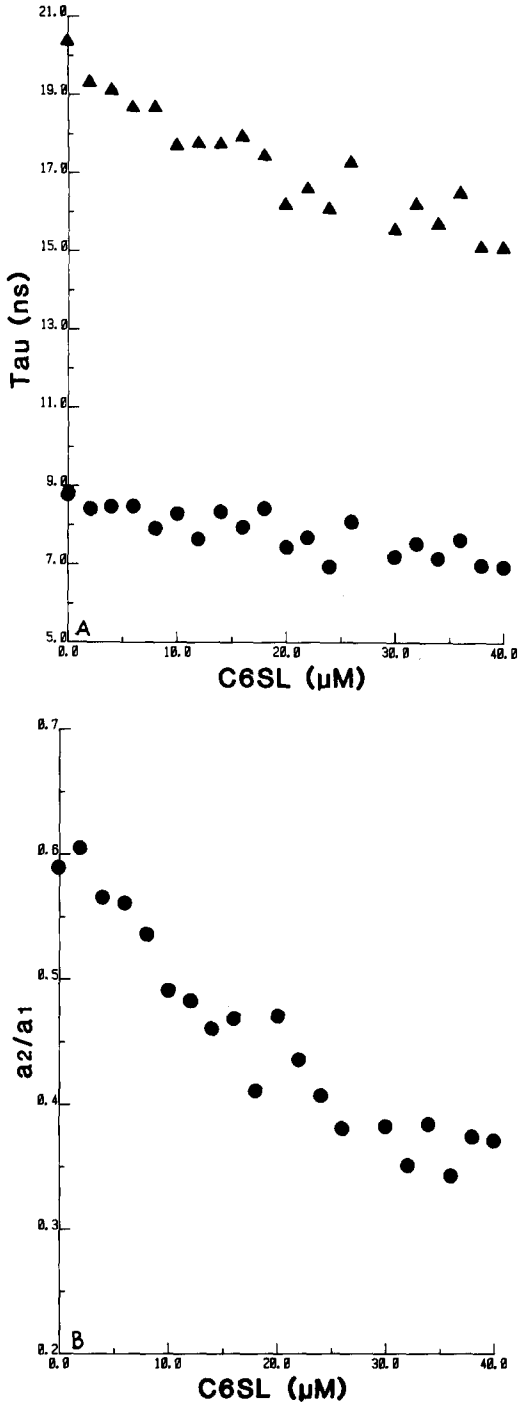

Fig. 5. (A) The effect of C6SL on the lifetimes τ_1 (●) and τ_2 (▲) of 6.6 μM ANS in the presence of erythrocyte ghosts, pH 6. (B) The effect of C6SL on the relative populations, a_2/a_1, of the two lifetime components in the presence of erythrocyte ghosts, pH 6.

populations of the two different lifetime species at zero time. The short lifetime component has a time constant of 8.9 ns and the long lifetime component has a time constant of 20.4 ns. The ratio a_2/a_1 equals 0.6. These values may vary slightly with different membrane preparations, buffer pH, ionic strength, and ANS concentration [11,18]. Consequently, in each of the experiments to be mentioned later, a control is used to establish a basis for comparison.

Quenching of membrane-bound ANS by C6SL

With the addition of C6SL to the erythrocyte membranes suspension, both the long and short lifetimes are decreased (Fig. 4, dotted curve). In this case, at pH 6 in the presence of 40 μM added C6SL, the lifetimes are reduced to 15.1 and 6.9 ns (25% and 22% quenching, respectively); the a_2/a_1 ratio is reduced to 0.37 (−37%).

An increase in pH resulted in the partial loss of C6SL quenching ability. At pH 9.5, 40 μM of C6SL reduces the lifetimes from 19.5 to 16.3 and from 8.3 to 7.1 (16% and 14% quenching, respectively). a_2/a_1 changes from 0.5 in the control to 0.33 (−34%) in the presence of the quencher.

Effects of C6 on membrane-bound ANS

C6 is the non-spin-labeled analogue of C6SL. At concentrations comparable to that used for C6SL, C6 has only a slight quenching effect on both lifetimes (about 5% for τ_2, for τ_1 at 40 μM C6). In contrast, the effect on the ratio a_2/a_1 is comparable to that created by C6SL (from 0.53 to 0.38, −28%).

Effects of C2SL on membrane-bound ANS

C2SL is an analog of C6SL with a two-carbon alkyl tail group. The effects of C2SL on the ANS lifetimes are negligible at 40 μM. At a concentration of 100 μM, τ_2 goes from 19.3 to 18.8 (−2.6%) and τ_1 goes from 8.8 to 8.4 (−4.5%).

Effects of C6SLMeI on membrane-bound ANS

C6SLMeI is a quaternary amine analogue of C6SL. Its effects on ANS fluorescence lifetimes are less pronounced but similar to those of C6SL. At pH 6, 40 μM C6SLMeI exhibits a 15% quenching on τ_2 and a 12% quenching on τ_1. The ratio of a_2/a_1 was reduced by 31%. When the experiment

TABLE II

EFFECT OF 40 μM OF LOCAL ANESTHETICS ON ANS FLUORESCENCE DECAY PARAMETERS IN ERYTHROCYTE GHOSTS

	C6SL		C6SLMeI		C2SL	C6
pH	6	9.5	6	9.5	6	6
τ_1	−23%	−14%	−12%	−10%	−3%	−1%
τ_2	−26%	−16%	−15%	−13%	0%	−5%
a_2/a_1	−37%	−32%	−31%	−23%	−5%	−28%

was repeated at pH 9.5, at 40 μM C6SLMeI, τ_2 is reduced by 13% and τ_1 by 9%. The concomitant change in a_2/a_1 is −23%. The results of the quenching studies on erythrocyte membranes are summarized in Table II.

Quenching of sarcoplasmic reticulum-bound ANS by C6SL

ANS binds to sarcoplasmic reticulum membranes and exhibits two lifetimes of 16.8 ns and 7.9 ns. The a_2/a_1 ratio is 0.32.

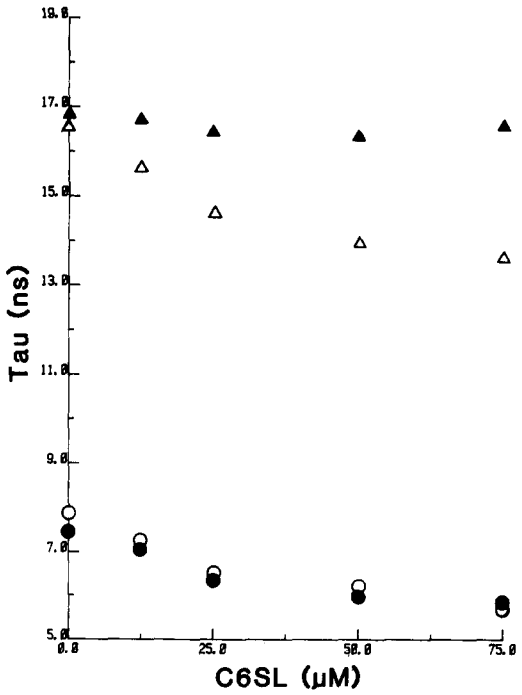

Fig. 6. Effect of C6SL on the lifetimes of 6.6 μM ANS in the presence of sarcoplasmic reticulum membranes. ▲, τ_2 at pH 9.5; ●, τ_1 at pH 9.5. Open symbols represent the respective components, but at pH 6.

The addition of C6SL to ANS-labeled sarcoplasmic reticulum suspension produced quenching consistent with that of ANS-labeled erythrocyte membranes. At 50 μM, pH 6, C6SL quenches τ_1 by 23%, τ_2 by 17%, and reduces a_2/a_1 by 26%. Fig. 6 shows the changes in lifetimes as functions of quencher concentration at pH 6 and pH 9.5.

At pH 9.5, the quenching effects of C6SL are diminished. At 50 μM, C6SL reduced τ_1 by 21% and τ_2 only by 3%. The change in a_2/a_1 is only 5%. Thus, in sarcoplasmic reticulum membranes, positively charged local anesthetics are much more effective quenchers of τ_2.

Discussion

ANS lifetimes

The single 7.6 ns ANS lifetime in the presence of PC/cholesterol liposomes is in very good agreement with previous results [18–21]. The observation of two ANS lifetimes, τ_1 and τ_2 of approx. 8 and 20 ns, in the presence of biological membranes is in agreement with published results from erythrocyte membranes [11,18,22] and from microsomal membranes of *Tetrahymena* [25]. τ_1 is similar to the lifetime measured in the PC/cholesterol and PC/PS liposomes. Fortes [18] observed the shorter lifetime in liposomes made from extracted erythrocyte membrane lipids. The longer lifetime, τ_2, is similar to lifetimes observed in proteins–for example, 14.8 to 16.7 ns for albumin [23–26]; 17.9 to 18.1 ns for dehydrogenase [27]; 16.4 to 18.7 ns for apomyoglobin [28,29]. Therefore, there is good support for assigning τ_1 to ANS located in the phospholipid bilayer region and τ_2 to ANS in regions associated with membrane proteins (e.g.,

regions of lipid-protein interactions). There are no published data for the lifetimes of ANS in sarcoplasmic reticulum membranes.

The two major lifetime components observed in the erythrocyte and the sarcoplasmic reticulum membranes probably are averages of lifetimes of ANS molecules having similar but not identical microenvironments. The short lifetime represents the average of the lifetimes of the ANS molecules in the lipid region and the long lifetime represents the average of the lifetimes of ANS molecules in the protein region.

Quenching results

The data clearly indicate that C6SL is a potent paramagnetic quencher of ANS fluorescence in the model systems as well as in biological membranes. The nonspin-labeled analog, C6, is not an effective quencher. It requires two orders of magnitude greater concentration to produce effects on both components of erythrocyte membrane-bound ANS fluorescence comparable to those of C6SL.

In micromolar concentrations, C6SL is capable of quenching the fluorescence of liposome-bound ANS, protein-bound ANS, and membrane-bound ANS. The nitroxide spin label on the anesthetic molecule is responsible for the quenching. Quenching of erythrocyte membrane-bound ANS by C6SL was first reported by our laboratory [11,30]. The mechanism may be enhanced intersystem crossing or enhanced internal conversion. Thus, for S_0, S_1, T_1, and Q representing ground and first singlet states, first triplet state, and quencher, respectively,

$$S_1 + Q \rightarrow T_1 + Q$$

or

$$S_1 + Q \rightarrow S_0 + Q.$$

Such quenching is diffusion-controlled with an interaction distance of 0.4 to 0.6 nm [31].

For the series of spin-labeled local anesthetics, quenching efficiencies are in the order C6SL > C6SLMeI > C2SL. This correlates with the partition coefficients estimated by electron spin resonance studies [34], and by ultraviolet light absorption studies on erythrocyte ghosts (Blickenstaff, unpublished). Thus, initially, the amount of quenching is dependent on the number of quenchers intercalated in the membrane, which in turn is a function of the partition coefficient.

Effects of pH on the quenching ability of C6SL

The quenching effect of C6SL on PC/cholesterol liposomes is independent of pH (from 6 to 9.5). The local anesthetic is less protonated at higher pH ($pK = 7.2$ to 7.4, Wang, H.H., unpublished results). This indicates that (1) C6SL does not rely on charge-pair formation with ANS to effect quenching since the uncharged form quenches equally well in liposomes, and (2) the amount of C6SL intercalated in the liposomal membrane is not measurably changed by pH, indicating that hydrophobic interaction is sufficient to hold this compound in the membrane. This is in agreement with earlier results from this laboratory showing that C6SL binds to PC liposomes primarily by hydrophibic interactions [34]. The results further indicate the the quenching process itself is not directly affected by pH changes.

Since C6SL is known to interact with acidic phospholipids electrostatically (Ref. 34 and Wang, H.H., unpublished), the experiments with mixed PC/PS liposomes were performed. The results were the same as with PC/cholesterol liposomes. ANS has a very low affinity for PS [36]. Hence, in such a pure model system of PC/PS liposomes, ANS is not necessarily distributed near any PS molecules and therefore can not be sensitive to any change in the PS-C6SL interactions effected by raising the pH. Furthermore, since C6SL can diffuse laterally in liposomes, whether protonated or unprotonated, its quenching of ANS is therefore independent of pH in lipid model systems.

The quenching effect of C6SL on erythrocyte membrane-bound ANS, however, showed some pH dependence. The quenching effect decreases with increasing pH which suggests some electrostatic interactions of C6SL in the erythrocyte membrane. At higher pH, less access of uncharged local anesthetic to the vicinity of ANS binding sites, including the protein regions, reduces contact quenching. The fact that the quenching cannot be totally abolished at high pH indicates that the local anesthetic C6SL has some access to both lipid and protein regions of the membrane regardless of its form, charged or uncharged. The loss of

charge on the local anesthetic must account for the observed changes because ANS lifetimes, in the absence of anesthetics, are not sensitive to pH changes.

In control experiments, the quenching effect of C6SLMeI (the permanently-charged quaternary analogue of C6SL) is not affected by pH. Again this demonstrates that C6SL results are not the consequence of the effects of pH on ANS or on the membrane.

The effects of cationic amphiphiles on relative distribution of ANS

The local anesthetics and other amphiphiles have a similar effect on a_2/a_1, the ratio of ANS in the protein and lipid regions. This may be accounted for by the increased binding of ANS to the membranes promoted by amphiphiles. Fortes [18] reported addition of divalent and trivalent cations resulted in increased binding but decreased τ values. Other have observed enhanced steady-state fluorescence under the same conditions [36–39]). When local anesthetics were added to membranes, the amount of membrane-bound ANS increased and ANS fluorescence was enhanced [11,21,40,41].

Since ANS is believed to be located near the polar headgroup region of model membrane systems [42–48], its interaction with the membrane is very dependent on the surface potential of the membrane. Rubalcava et al. [38] found that ANS bound more tightly to neutral or cationic detergents than to anionic detergents. Addition of organic anions such as tetraphenylborate, which partitions into the membrane produces a bound negative charge, decreases the binding of ANS but not the quantum yield of bound ANS [39]. The ANS ions that intercalate in the membrane make the surface potential more negative. At high ANS concentrations, the electrostatic potential becomes sufficiently large so that the binding appears to be saturated. This apparent saturation occurs far below the true saturation level of ANS binding to membrane. Under these conditions the binding of ANS to different regions of the membrane is determined essentially by the surface potential and not by the number of available sites. Addition of cations or amphiphiles such as local anesthetics, or increasing the ionic strength, reduces the surface potential as predicted by the Gouy-Chapman double-layer theory. Since the sites are far from true saturation, charge screening allows more ANS to bind to the membrane and the fluorescence increases.

If we assume that there are fewer protein binding sites than lipid binding sites for ANS on the membrane but the affinity of protein binding sites for ANS is greater, at low ANS concentrations the probe would bind at first to the proteins and later to the lipids [49–51]. The concentrations of ANS used in these experiments are low (6 to 10 μM). If the effect of the added amphiphiles were to increase ANS binding, subsequent binding of more ANS in the lipid region would change the relative population of the bound ANS, decreasing the ratio of a_2/a_1. Therefore, the change in this ratio reflects the amphiphile's ability to alter the membrane surface potential.

Effects of C6SL on sarcoplasmic reticulum-bound ANS parameters

C6SL quenches the fluorescence of membrane-bound ANS in the sarcoplasmic reticulum. The effect of quenching is greater on τ_1 than on τ_2 for the concentration range tested. For example, τ_1 and τ_2 are reduced by 23% and 17%, respectively, at a C6SL concentration of 50 μM. However, at high pH, the quenching of τ_2 is almost abolished. Thus it appears that, for the sarcoplasmic reticulum membrane, the quenching of τ_2 is highly dependent on the positive charge of the anesthetic. The major protein component (up to 80%) in the sarcoplasmic reticulum membrane is (Ca^{2+} + Mg^{2+})-dependent ATPase [52]. An electrostatic interaction of this major protein with the tertiary amine local anesthetic would account for the large pH dependence of fluorescence quenching by C6SL in the sarcoplasmic reticulum. On the other hand, the erythrocyte membrane, with its heterogeneous protein composition, showed a smaller pH dependence in the quenching of τ_2 by C6SL. This result suggests that a smaller proportion of erythrocyte membrane proteins interact electrostatically with local anesthetics.

Our results support the concept of multiple binding sites of local anesthetics in membranes. Local anesthetics partition into the membrane by intercalating into lipid bilayer with the polar amine

headgroup located near the polar interface of the membrane. Such intercalation provides the proximity favorable for ANS quenching since ANS is believed to intercalate at the polar-apolar interface of the membrane. Local anesthetics, once intercalated into the bilayer, may diffuse laterally and thus interact with membrane components, lipids as well as proteins. Such interaction is inferred from the quenching by spin-labeled anesthetics of ANS lifetimes corresponding to protein and lipid regions. Although both the protonated and unprotonated forms of local anesthetics (C6SL) are equally good quenchers of liposome-bound ANS, there are important pH-dependent differences in the quenching of ANS associated with membrane proteins; this finding is particularly obvious in the sarcoplasmic reticulum membrane. These results lead us to suggest that protein-anesthetic interactions in biological membranes are dependent upon the anesthetic charge and the ionic character of the membrane proteins.

Acknowledgments

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