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Spin label study of local anesthetic–lipid membrane interactions. Phase separation of the uncharged form and bilayer micellization by the charged form of tetracaine

Wilson A. Frezzatti, Jr., Wilson R. Toselli and Shirley Schreier *

Laboratory of Molecular Biophysics, Department of Biochemistry, Institute of Chemistry, University of São Paulo, C.P. 20780, CEP 01498, São Paulo S.P. (Brazil)

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The interaction between tetracaine and egg phosphatidylcholine (egg PC) multibilayers was examined. ESR spectra of an ester spin label indicate that at low uncharged anesthetic : lipid ratios, membrane organization decreases. At higher ratios, saturation and phase separation occur, as suggested by a second spectral component which appears when the water solubility of tetracaine is reached. However, experiments with the drug in the absence and in the presence of membranes, making use of a phospholipid spin label, suggest that the new phase does not consist of solid tetracaine alone. Location of the new phase in the membrane would require a change in partition coefficient, while its location outside would imply a mechanism whereby the anesthetic would come off the membrane as an aggregate containing spin probe and phospholipid. Charged tetracaine forms micelles which disrupt-unilamellar egg PC vesicles (Fernandez, M.S. (1981) Biochim. Biophys. Acta 646, 27–30). Micellar tetracaine added to bilayers containing a PC spin probe changes the spectrum from one typical of a bilayer into one typical of micelles, indicating the formation of a tetracaine-egg PC mixed micelle. The effect is reversible upon dilution to concentrations below the critical micelle concentration of tetracaine. When membranes are prepared in the presence of a water-soluble spin label, TEMPOcholine, ascorbate destroys the signal of untrapped label; when mixed phospholipid-tetracaine are formed by addition of micellar tetracaine, this leads to a complete loss of the ESR signal. High drug concentrations are often used for anesthesia and could be related to morphological nerve damage caused by large doses of anesthetics.

* To whom correspondence should be addressed.

Abbreviations: PC, egg phosphatidylcholine; 5-SASL, stearic acid containing the 2′,2′-dimethyl-*N*-oxyl oxazolidine moiety at carbon 5; 5-MeSL, methyl ester of 5-SASL; 12-PCSL, dipalmitoylphosphatidylcholine containing the *N*-oxyl oxazolidine moiety at carbon 12 of the acyl chain at position *sn*-2; 5-PCSL, dipalmitoylphosphatidylcholine containing the *N*-oxyl oxazolidine moiety at carbon 5 of the acyl chain at position *sn*-2; ASL and CSL, 2′,2′-dimethyl-*N*-oxyl oxazolidine derivatives of 5α-androstan-17β-ol-3-one and cholestan-3-one, respectively; TEMPOcholine, choline cation where one methyl group was replaced by the 4-(2,2,6,6-tetramethyl-*N*-oxyl)piperidinyl moiety; cmc, critical micelle concentration.

Introduction

The mechanism of action of local anesthetics is thought to involve crossing of the membrane by the uncharged form and binding of the charged form to a specific site of the sodium channel located near the inner surface of the nerve membrane [1]. Nevertheless, in many cases large doses are required for anesthesia [2], and a large amount of work has been done showing that such concentrations, or smaller ones, are capable of alter-

ing both the permeability and the structure of lipid membranes [3–6].

Magnetic resonance techniques have been used to investigate the effect of local anesthetics on the organization of membrane lipids. Spin-label ESR [4,7–9], proton [10–12], deuterium [5,6,13–16], and phosphorus [5,17,18] NMR spectra have provided information about local anesthetic-membrane interaction at a molecular level.

When the membranes consist of egg PC, it was found that the binding of the local anesthetic tetracaine varies with anesthetic charge, both qualitatively and quantitatively [14]. The amounts of charged and uncharged anesthetic in the aqueous and membrane phases depend on membrane concentration [9] and it is important to take this fact into account when analysing the effect of either form upon membrane structure and function.

In the present work we analyse the effect of the local anesthetic tetracaine upon the ESR spectra of spin probes intercalated in egg PC bilayers. At sufficiently high concentrations and at high pH, the uncharged form of tetracaine saturates the membrane and phase separation occurs upon further addition of the anesthetic.

The charged form of tetracaine forms micelles (cmc = 70 mM, Ref. 19) capable of disrupting egg PC unilamellar vesicles as shown by light scattering and by phosphorus determination of filtrates [20]. Making use of spin probes, we provide spectroscopic evidence for the incorporation of the phospholipid into tetracaine-egg PC mixed micelles and we demonstrate the reversibility of the phenomenon. Also, by using a water-soluble spin label, we demonstrate the loss of the aqueous compartments of uni or multilamellar egg PC membranes upon addition of micellar tetracaine.

A preliminary account of some of the present results has appeared elsewhere [8].

Materials and Methods

Tetracaine and dicetyl phosphate were obtained from Sigma Chemical Co, St. Louis, MO; 5-MeSL and 5-SASL came from Syva, Palo Alto, CA. 12-PCSL was from Serdary Research Laboratories, London, Ontario; 5-PCSL and TEMPO-choline chloride were prepared by P. Laks from Simon Fraser University, Burnaby, British Columbia, and were generous gifts of Dr. I.C.P. Smith from the National Research Council of Canada. Egg PC was prepared by the method of Nielsen [21]. All other reagents were analytical grade.

A 0.12 M borate/phosphate/citrate buffer was used throughout. Samples were prepared by evaporating $CHCl_3$ stock solutions of egg PC and spin labels under wet nitrogen. Unless otherwise stated, the lipid to probe ratio was 100 : 1 on a molar basis. The samples were placed under vacuum for no less than 2 h, then hydrated with buffer at the desired pH. Whenever addition of tetracaine altered the pH it was adjusted by adding concentrated acid or base. pH mesurements were done on a Metrohm E 512 pH-meter. ESR spectra were run on an E-4 Varian ESR spectrometer, at room temperature (22 ± 2°C). Samples were placed in flat quartz cells for aqueous solutions from James Scanlon, Costa Mesa, CA.

Results

The present work tries to distinguish effects caused by the uncharged form of tetracaine from those caused by the charged form. As calculated from the model described in Ref. 9, at pH 10.5 most of the anesthetic is in the uncharged form,

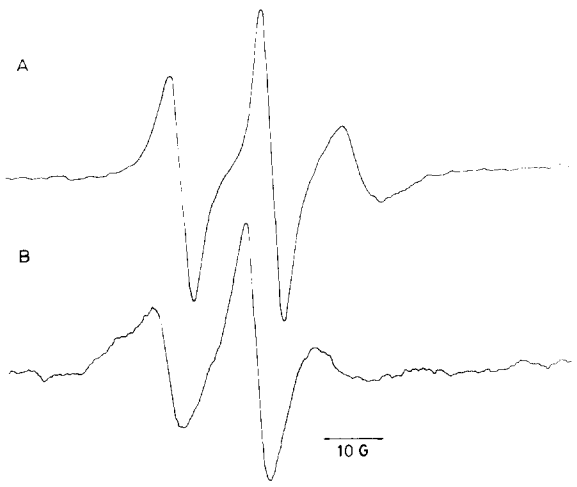

Fig. 1. ESR spectra of 5-MeSL in 5.2 mM egg PC membranes, pH 10.5 (A) In the presence of 2.3 mM tetracaine. (B) In the presence of 20 mM tetracaine. In the absence of tetracaine, the lineshape is similar to that in (A) [8,9].

the amount of charged form being negligible. The converse is true at pH 5.5.

Membrane saturation and phase separation by the uncharged form of tetracaine

Fig. 1A shows the ESR spectrum of 5-MeSL in 5.2 mM EPC at pH 10.5. We have previously justified [9] the use of the empirical parameter h_{+1}/h_0 (ratio of heights of the low field to mid-field lines) as a measure of the combined effects of tetracaine on the degree of lipid order and mobility (see Discussion). At low anesthetic-to-phospholipid ratios, the h_{+1}/h_0 ratio increases with increasing pH, until a plateau is reached (Fig. 2A and Ref. 9), indicating a decrease in order and/or increase in mobility caused by increased partitioning of the uncharged form of tetracaine.

This increased partitioning is a result of the shift in equilibrium towards the uncharged form with increasing pH. Since the partition coefficient for this form is much larger than that for charged tetracaine [9,14], increasing amounts of uncharged tetracaine partition into the membrane with increasing pH [9].

When the experiment was performed at higher anesthetic : phospholipid ratios, at pH 10.5 spectra like that in Fig. 1B were obtained, where an additional component is observed. The contribution of this spectral component, seen as a distinct shoulder at low field in Fig. 1B, increases as the tetracaine : egg PC ratio increases. Due to its lineshape, the new spectral component contributes to an actual decrease of the h_{+1}/h_0 ratio, and this is seen in Fig. 2B. We ascribe the appearance of the new spectral component to the following. At low tetracaine : egg PC ratios, the uncharged form of the anesthetic is solubilized by the bilayer; after reaching a critical drug : lipid ratio (see below) additional anesthetic goes into a new phase which gives rise to a different spectrum.

In order to verify whether solid uncharged tetracaine could incorporate 5-MeSL, experiments were performed in the absence of membranes. When buffer containing the solid drug was put in contact with a film of 5-MeSL made on the wall of a test tube, either no spectrum or a very exchange-broadened one was obtained, indicating that a solid tetracaine phase is largely unable to accommodate 5-MeSL. Similar results were obtained when the probe was added from a concentrated methanol solution. Experiments were done at variable membrane concentrations. ESR spectra of 5-MeSL were obtained for various egg PC concentrations and increasing tetracaine : egg PC molar ratios. These ratios were calculated for membrane-located uncharged anesthetic from the partition coefficient in Ref. 9. Fig. 3 shows that

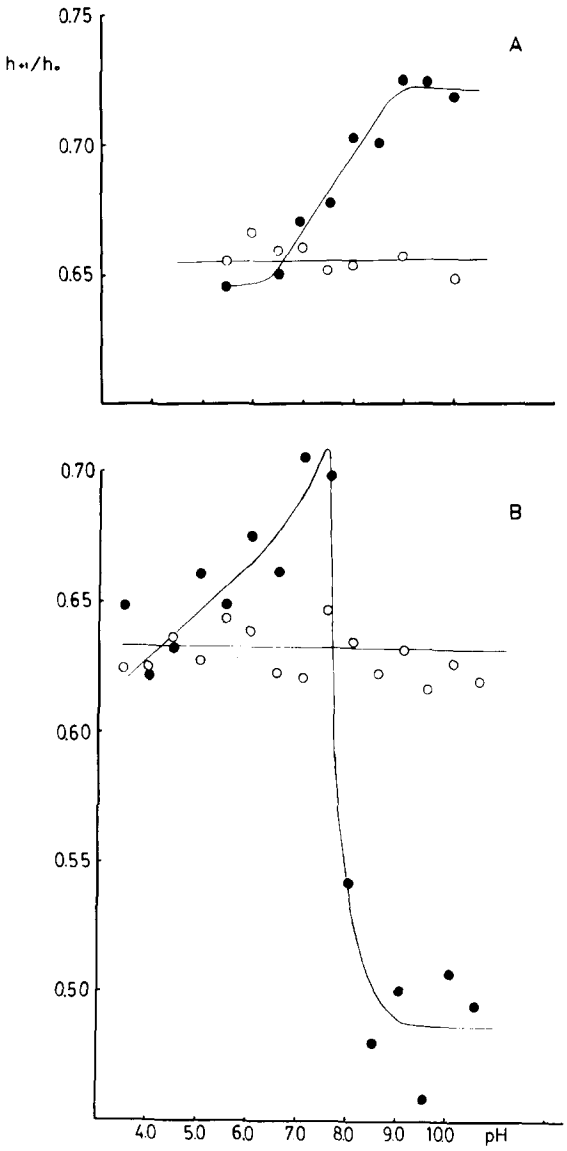

Fig. 2(A) and (B). h_{+1}/h_0 ratio as a function of pH for the samples of Fig. 1 A and B, respectively (●) and for controls in the absence of tetracaine (○). (Fig. 2A is taken from Ref. 9, with permission).

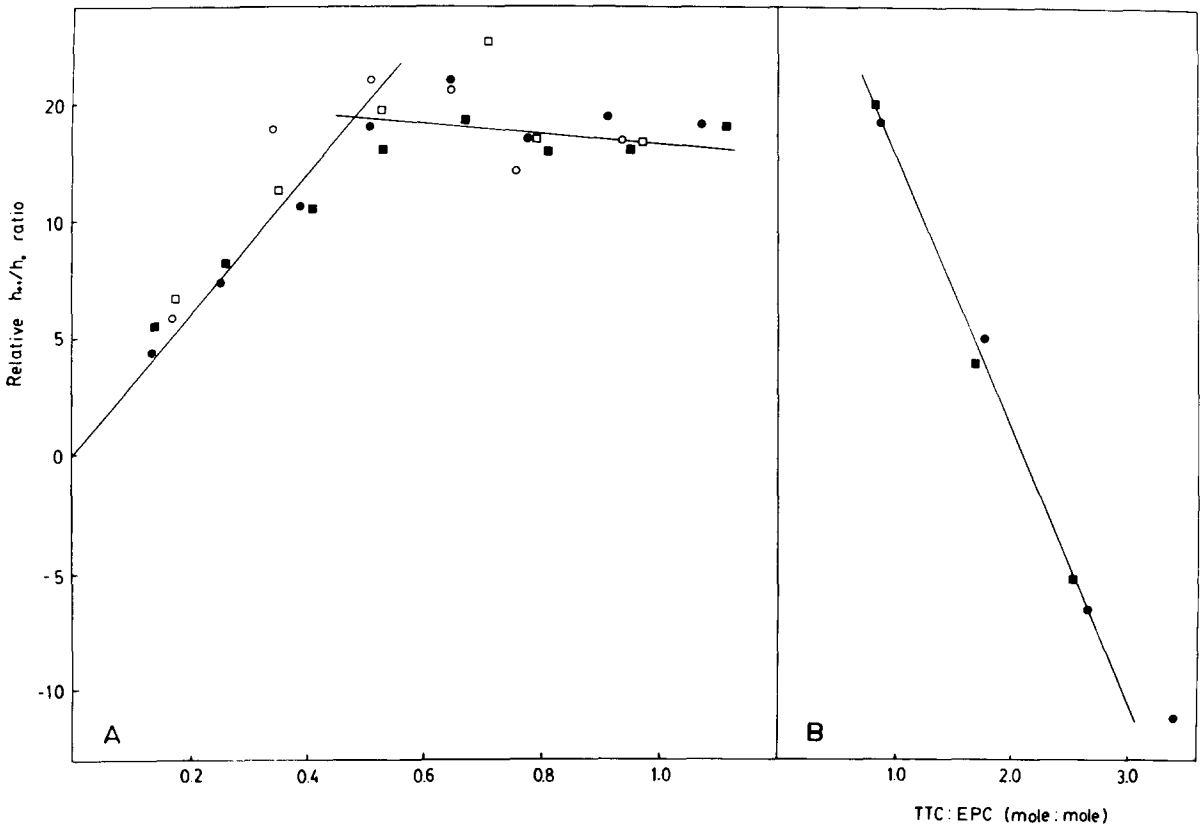

Fig. 3(A) and (B). Relative h_{+1}/h_0 ratio as a function of tetracaine : egg PC mole ratio for two samples containing 16 mg egg PC/ml (○, ●), and two samples containing 32 mg egg PC/ml (□, ■), pH 10.5. The anesthetic-to-lipid ratio was calculated from tetracaine concentrations in the membrane, estimated from the partition coefficient in Ref. 9.

the h_{+1}/h_0 ratio depends on the anesthetic : lipid ratio: the h_{+1}/h_0 ratio first increases, as a result of increased anesthetic partitioning. Then, a break occurs and, at tetracaine : egg PC > 1, h_{+1}/h_0 decreases sharply. We ascribe the break to the onset of the second spectral component, i.e., the appearance of the second phase. The molar ratio at which the breaks were observed varied from about 0.55–0.65:1 tetracaine : egg PC.

In order to check for the possibility and extent of phospholipid incorporation in the newly formed phase, experiments were performed using a phospholipid spin probe, 12-PCSL, at a high label : egg PC molar ratio (16 : 100), so that an exchange-broadened ESR spectrum was obtained (Fig. 4). The c/d ratio, mesured on the low field line, indicates the extent of exchange broadening which is a result of the rate of collision between labeled molecules and should decrease with decreasing concentrations of the latter. Phospholipid is effectively diluted upon anesthetic partitioning into the membrane and Fig. 5 shows that the c/d ratio decreases steeply for increasing tetracaine : egg PC ratios up to about 0.65 : 1. Higher ratios lead to a much smaller decrease of c/d.

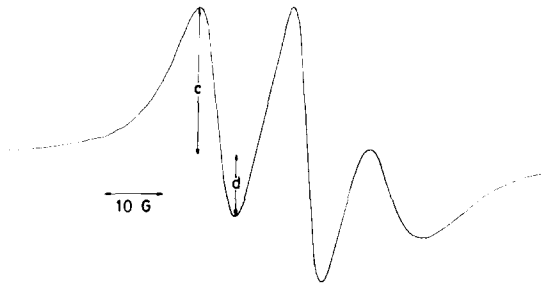

Fig. 4. ESR spectrum of 12-PCSL (14 mol%) in 5.2 mM egg PC membranes, pH 10.5. The extent of exchange interaction was estimated from the c/d ratio.

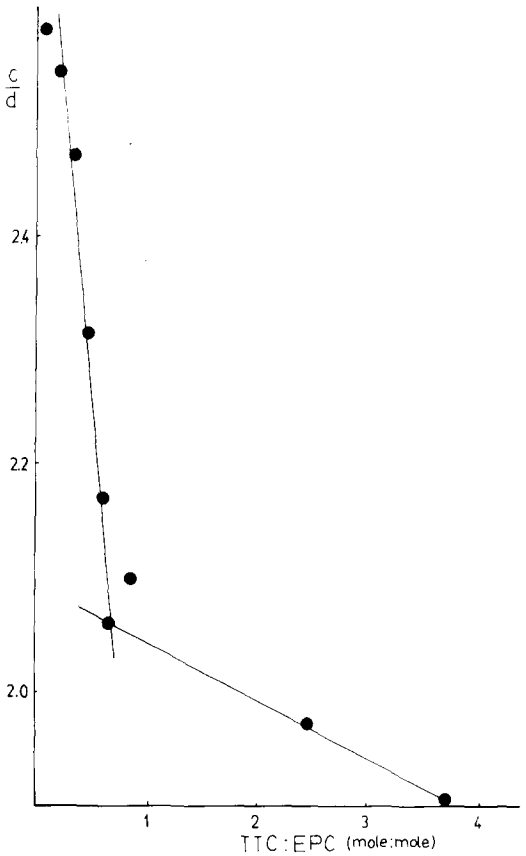

Fig. 5. c/d ratio as a function of tetracaine:egg PC molar ratio for the sample in Fig. 4. The anesthetic to lipid ratio was calculated as in Fig. 3.

Phospholipid bilayer disruption by micellar charged tetracaine: reversibility of the phenomenon

Fernandez has reported that charged tetracaine forms micelles with a cmc of about 70 mM [19]. She has also shown that when micellar tetracaine was added to egg PC unilamellar vesicles at a 100 : 1 anesthetic-to-lipid molar ratio, light-scattering measurements and phosphorus determination of filtrates gave an indication of membrane disruption by the micellized anesthetic.

The experiments below were designed to provide spectroscopic evidence for phospholipid incorporation into a charged anesthetic-egg PC mixed micelle.

When 5-PCSL was incorporated into egg PC multibilayers, a spectrum typical of a membrane structure was obtained (Fig. 6A). Such spectrum resembles very closely that yielded by the fatty

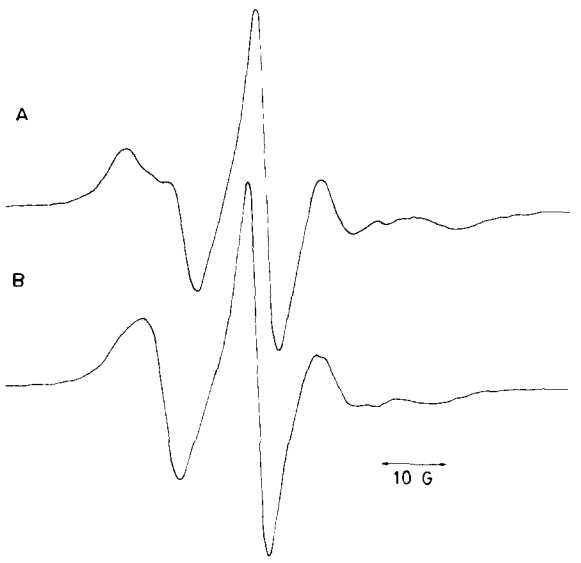

Fig. 6. ESR spectrum of 5-PCSL in 5.2 mM egg PC (pH 6.2). (A) In the absence of tetracaine. (B) In the presence of 80 mM tetracaine. When 5-SASL was used instead of 5-PCSL, the results were very similar.

acid alone (5-SASL). Spectra of the latter probe in micellar detergents are well known [22]. When micellar tetracaine is added to egg PC containing 5-PCSL, the spectrum (Fig. 6B) is very similar to that obtained for micellar systems [22], as well as that obtained for micellar tetracaine alone (not shown). The results clearly indicate the formation

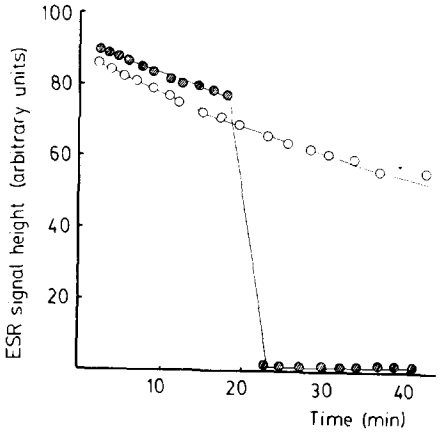

Fig. 7. Kinetics of the decay of the ESR signal height of a 1.1 mM TEMPOcholine solution in 5.2 mM egg PC containing 4 mol% dicetyl phosphate (pH 6.2). The signal decay was monitored after addition of 4.1 mM ascorbate (final concentration). After 20 min, either buffer (○) or 92 mM tetracaine (final concentration) (●) was added.

of a mixed tetracaine-egg PC micelle. Similar results were obtained both for unilamellar and multilamellar egg PC bilayers; membrane disruption was observed for egg PC: tetracaine molar ratios up to 14 : 100.

Dilution of the mixed micelles to tetracaine concentrations below the cmc led to complete recovery of the bilayer spectrum, indicating that membrane disruption is reversible.

Membrane disruption was also demonstrated by the loss of the aqueous compartments trapped in egg PC multibilayer dispersions. Experiments were performed according to Aracava et al. [23], by preparing the membrane in buffer containing the water-soluble spin label, TEMPOcholine chloride. Addition of ascorbate to the bulk aqueous phase leads to an immediate loss of the TEMPOcholine signal due to label in this phase and a slow decrease of the signal due to label remaining in the bilayer-enclosed water compartments (Fig. 7, open symbols). When micellar tetracaine is added (Fig. 7, solid symbols), an instantaneous loss of the remaining ESR signal occurs, indicating the complete loss of the trapped water compartments.

Discussion

Deuterium NMR studies [5] have shown that when uncharged tetracaine partitions into egg PC bilayers, there is a decrease in the bilayer degree of order.

Ester spin probes like 5-MeSL are weakly anchored at the membrane surface and therefore possess a high degree of motional freedom. For that reason, it has been common practice in the literature to calculate rotational correlation times from ESR spectra of these probes. However, as discussed by Cannon et al. [24] and Schreier et al. [25], as these probes undergo some degree of ordering in a membrane bilayer, it is not correct to extract quantitative information about their rate of motion from their ESR spectra. Thus, we have used changes of the empirical h_{+1}/h_0 parameter as a monitor of the combined changes in the degree of order and mobility of the probe 5-MeSL upon addition of uncharged tetracaine to egg PC membranes.

Taking the present, and deuterium NMR data [5] in conjunction, we can say that uncharged form of tetracaine affects order (decreases) and, most likely, mobility (increases), of egg PC bilayers at low anesthetic phospholipid ratios.

As the tetracaine : phospholipid ratio increases, a second spectral component appears (Fig. 1B). This component could be due to membrane saturation by the anesthetic and formation of a second phase which could be located either in the membrane, or in the aqueous phase, or in both.

The experiments performed at different membrane concentrations (Fig. 3) indicate that the break in the plots of h_{+1}/h_0 vs. tetracaine : egg PC occurs at molar ratios of about 0.55–0.65 : 1. Making use of the partition coefficient for uncharged tetracaine in egg PC membranes [9] it is found that the 0.65 : 1 ratio leads to an anesthetic aqueous concentration which corresponds to its solubility in this medium (about 1.2 mM, Boulanger, Y., Ph.D. thesis; Ref. 26). This would suggest that the second spectral component would arise from 5-MeSL intercalated in a solid tetracaine phase which starts to form at the water solubility of the anesthetic, leading to no further partitioning into the membrane. That is, membrane saturation by the drug is imposed by a combination between the partition coefficient and its water solubility, as observed with micellar systems [27] and in studies of the effects of aliphatic alcohols [28] and an antichagasic drug (Holland, M.A.G.L. and Schreier, S., unpublished data) upon phospholipid membranes.

Nevertheless, attempts to incorporate 5-MeSL into solid uncharged tetracaine in buffer, in the absence of membrane indicated the lack of ability of the anesthetic to uptake the probe (see Results).

Moreover, incorporation of 5-MeSL previously in the membrane into the newly formed solid tetracaine phase would require transfer from the former to the latter via the aqueous phase; this would not be a viable mechanism, since it seems to depend on the probe partitioning into water [29]. We have previously observed that when a detectably water-soluble probe, ASL, was incorporated in a lipid membrane, there was a time-dependent transfer of the probe to aggregated amphotericin B, with a corresponding change in the spectral lineshape. However, when the membrane was previously labeled with a water-insoluble probe, CSL, no transfer was detected [29].

Since 5-MeSL is highly water-insoluble, it is very unlikely that its transfer from the membrane to a phase located outside would be mediated by the aqueous medium.

Finally, the results obtained with the phospholipid spin probe, 12-PCSL, suggest that the new phase contains, in addition to spin probe, a small amount of phospholipid (Fig. 5).

Whether this phase is located in the membrane or not, is not possible to establish from the present experiments. Membrane location would mean that at least two phases with different anesthetic: phospholipid ratios coexist, and would require an overall change in the partition coefficient value. Changes in partition coefficient with increased partitioning have been reported for aliphatic alcohols in detergent micelles [30]. On the other hand, for the probes previously in the membrane to be transferred to a separate phase, this would require a mechanism not mediated by water. Such mechanism could be based on the equilibria

$$TTC_{solid} \rightleftharpoons TTC_{H_2O} \rightleftharpoons TTC_{membrane} \rightleftharpoons (TTC + probe + phospholipid)$$

whereby tetracaine (TTC) would come off the membrane as an aggregate containing also probe and a small amount of phospholipid, as suggested by the experiments with spin-labeled phospholipid. This would be consistent with the lack of 5-MeSL uptake by solid tetracaine. Analogous events have been described for solutes partitioning into detergent micelles [31].

As for the charged form of tetracaine, our results agree with the work by Fernandez [19] that shows that this species is capable of forming micelles which have the ability to disrupt lipid bilayers. We obtained spectroscopic evidente for the incorporation of egg PC into tetracaine-phospholipid mixed micelles and for the reversibility of this phenomenon upon dilution of tetracaine to concentrations below its cmc. We also demonstrate the loss of the aqueous compartments trapped by the egg PC bilayers (Fig. 7).

Local anesthetics have been previously shown to interact with lipid membranes, affecting bilayer organization and permeability. Local anesthetics have also been shown to influence bilayer to hexagonal phase transition and have been seen to promote membrane fusion [18,32–34]. Bilayer disruption by the charged form of tetracaine has also been reported [19,20].

The present experiments show that local anesthetics in their uncharged form can saturate the membrane and give rise to a new phase, while the charged form can reversibly incorporate bilayer phospholipid into mixed anesthetic-lipid micelles. It is worthwhile recalling that high drug doses are sometimes required for local anesthesia; thus, the events reported in the studies with model membranes could occur in the in vivo situation. Morphological observations have indicated that 2% tetracaine solutions (67 mM) can cause great nerve damage [35]. Also, other local anesthetics, when administered at high concentrations were seen to cause severe myelin degeneration [36]. It is noteworthy that in both cases the anesthetics were administered in ways that did not alter their initial concentration. We suggest that the observed morphological damage could be a result of membrane disruption caused by micellar anesthetic.

Acknowledgements

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