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USE OF MEMBRANE SPIN LABEL SPECTRA TO MONITOR RATES OF REACTION OF PARTITIONING COMPOUNDS; HYDROLYSIS OF A LOCAL ANESTHETIC ANALOG

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ESR spectra of membrane spin probes are conventionally used to obtain structural information. Here we show, for the first time, that when a membrane-soluble compound undergoes a chemical reaction, time-dependent changes in the ESR spectra of membrane spin probes can yield information about the kinetics of reaction. A benzoic acid ester, analog of the local anesthetic tetracaine, partitions between aqueous and membrane phases, causing changes in membrane structure as monitored by the ESR spectra of a probe. At alkaline pH, the lineshapes are time-dependent and the spectra go back to that in the absence of drug. The changes follow pseudo-first order kinetics. This effect is due to drug hydrolysis leading to water-soluble products, as confirmed by direct spectrophotometric measurements of the reaction. The pseudo-first order rate constants found by the latter method are in very good agreement with those calculated by ESR. The rate of hydrolysis decreases with increasing membrane concentration. This phenomenon accounts in part for the increased potency and toxicity of the more membrane-soluble local anesthetics.

ESR spectra of membrane spin labels are conventionally employed to analyse structural properties of membrane components (usually lipids) (1). A large body of work has been done examining the effects caused by membrane-soluble pharmacologically active drugs upon the organization and dynamics of biological membranes and lipid bilayers. Among these, local anesthetics have been widely examined (2-8). In conjunction with other techniques, in particular deuterium NMR (9-15), it has been found that the charged and uncharged forms of local anesthetics partition into membranes to a

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different extent (9), are located in different regions of the bilayers (3,10,14), and change bilayer organization in different ways (3,6,10). In addition, it has been shown by ESR that the apparent pK of tetracaine (TTC) (3,4) and some of its analogs (7,8) is a function of membrane concentration. Moreover, the charged form of TTC (3) and the uncharged form of some of its analogs (7,8) form micelle-type aggregates which disrupt lipid bilayers. Membrane saturation by uncharged TTC, followed by formation of a new phase, has been demonstrated (3,6).

Previous work from this laboratory has changed the usual focus of spin label experiments demonstrating that instead of asking how drugs affect membrane organization, one can inquire how membranes affect basic thermodynamic properties of drugs, such as ionization constants (3,4,7,8).

Here we show, for the first time, that time-dependent changes in the ESR spectra of a spin probe intercalated in the bilayer can be used to monitor the kinetics of reaction of partitioning compounds. This approach allowed the examination of the rate of alkaline hydrolysis of a TTC analog, as well as the role of membrane concentration upon this process.

EXPERIMENTAL PROCEDURES

Egg phosphatidyl choline (EPC) was obtained according to refs. 16 and 17. Spin label (5-doxyl stearic acid methyl ester, 5-MeSL) was from Sigma, St. Louis, MO. Cl-TTC, an analog of TTC bearing a chlorine atom at the para position, was prepared by a modification of the procedure in ref. 18, and characterized by IR and NMR spectra (G. Capobiano and A.T. do Amaral, in preparation). A 0.12 M phosphate-borate-citrate buffer (PBC, pH 11.5) was used throughout. All other reagents were analytical grade.

ESR spectra were run in flat quartz cells for aqueous solution from James Scanlon (Costa Mesa, CA) in a Varian E-4 spectrometer at room temperature ($22\pm2^{o}C$). Optical spectra were obtained in a Varian DMS-70 spectrophotometer at room temperature, pH measurements were done with a Metrohm E-512 pH-meter.

Lipid bilayers were prepared by evaporating chloroform solutions of EPC plus spin label (1 mole %) under nitrogen and leaving the samples under vacuum for no less than two hours. Multilamellar liposomes were formed upon addition of buffer and shaking in a vortex mixer for 5 min. The reaction mixtures were placed in a thermostated bath (37.0±0.5^{o}C) and aliquots were drawn for ESR and optical measurements. Spectrophotometric determination of hydrolysis was performed by stopping the reaction upon addition of acidic (final pH 6) Triton X-100 (1%, final concentration) to the reaction mixture. Controls contained EPC only. Samples were diluted in order to read the ester concentration at λ=246 nm (ε=13,500). Although this is not the maximum absorption wavelength (244 nm, ε=18,500), it was chosen to avoid overlaped absorption of the free benzoate.

RESULTS

Figure 1 shows the spectrum of 5-MeSL in EPC bilayers. Due to lack of strong anchoring to the aqueous interface, the spectra display a small degree of anisotropy. Since it is unreliable to extract either order parameters or correlation times from such spectra (19), we measured the empirical h_{+1}/h_0 parameter, as indicated in Figure 1. This parameter should be understood as providing information about the overall degree of membrane organization (6). Further discussion about the interpretation of h_{+1}/h_0 is given in ref. 6.

Addition of uncharged Cl-TTC leads to an increase in h_{+1}/h_0, indicating a decrease in membrane organization. Mean values of h_{+1}/h_0 (±S.E.) in the absence and presence (at time zero) of Cl-TTC were, respectively, 0.521 (±0.011) and 0.590 (±0.008) for 7.8 mM EPC and 3.8 mM Cl-TTC (seven

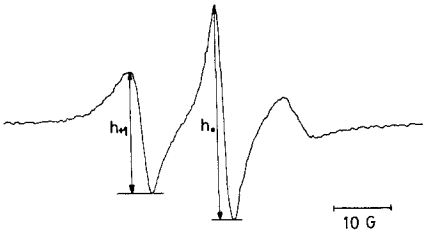

Figure 1. ESR spectrum of 5-MeSL in 7.8 mM EPC multibilayers, in 0.12 M PBC buffer. The figure shows how h_{+1} and h_0 are measured.

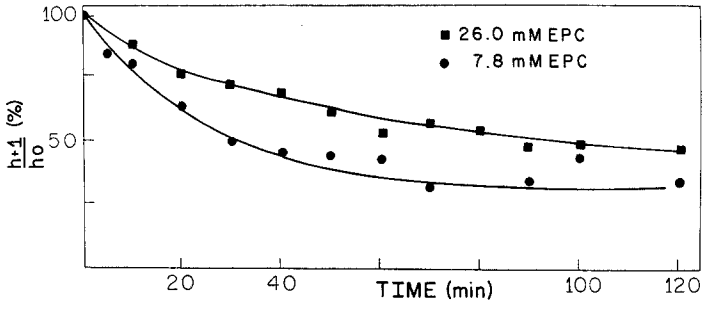

Figure 2. Representative experiment of the time-dependent effect of Cl-TTC upon the h_{+1}/h_0 ratio in the spectra of 5-MeSL. (■) 3.8 mM and (●) 9.30 mM Cl-TTC were added to 7.8 and 26.0 mM EPC membranes, respectively. See text for experimental details.

experiments), 0.519 (±0.008) and 0.600 (±0.009) for 13.0 mM EPC and 5.4 mM Cl-TTC (ten experiments), and 0.489 (±0.008) and 0.542 (±0.008) for 26.0 mM EPC and 9.3 mM Cl-TTC (ten experiments). Figure 2 indicates that the effect is time-dependent, showing a tendency of the system to return to the original h_{+1}/h_0 value (that in the absence of drug). Time-dependent changes are not observed in control samples (no drug added) suggesting that no hydrolysis of either EPC or spin label occurs in the time scale of the experiment. The curves can be analysed in terms of pseudo-first order kinetics and rate constants ($k\psi_m$) can be determined. Figure 3 (left) displays the values of $k\psi_m$ as a function of membrane concentration.

The results are suggestive of hydrolysis taking place, giving rise to products (a benzoate anion and an alcohol) that are more water-soluble than the parent compound and depleting the membrane of the structure-altering agent. To check this possibility, both freshly drawn samples and the same samples used in ESR experiments were analysed spectrophotometrically for the occurrence of hydrolysis, as described in the experimental section. It was found that Cl-TTC undergoes hydrolysis in a membrane concentration-

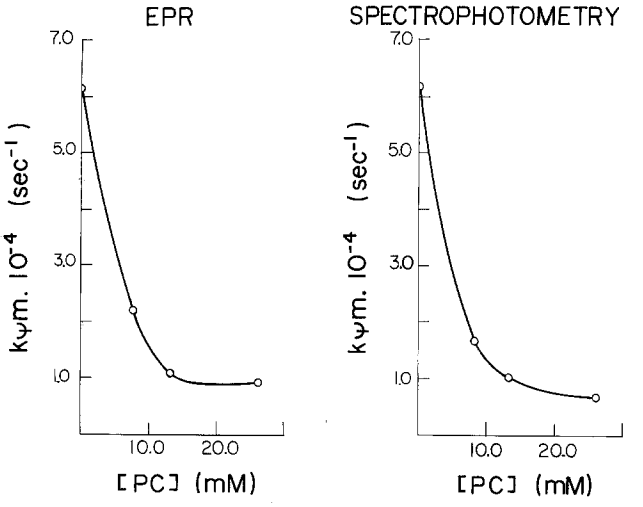

Figure 3. Experimentally determined pseudo-first order rate constants as a function of membrane concentration. ESR (left) and spectrophotometric (right) determinations. pH 11.5, 37±0.5°C. In the absence of EPC, $k\psi_m$ was determined only by spectrophotometry.

dependent fashion. The pseudo-first order rate constants are plotted in figure 3 (right) and display very good agreement with the results found by ESR, indicating that the latter is actually monitoring hydrolysis.

DISCUSSION

Cl-TTC partitions into the EPC multibilayers and equilibration between bulk aqueous phase, bilayers and internal aqueous layers is fast, as indicated by the immediate change in the ESR spectra. At alkaline pH, this change tends to revert, and analysis of the time-dependence of this process allows the calculation of the pseudo-first order rate constant of drug hydrolysis, as confirmed by direct spectrophotometric measurements. The ESR study was made possible by the fact that the products are more water-soluble than the parent compound leading to an effective loss of drug from the membrane.

A quantitative analysis indicates that the reaction takes place only in the aqueous phase, the role of the

membrane being that of protecting the drug from hydrolysis (Bianconi, Amaral and Schreier, submitted).

The more membrane-soluble, the more potent and toxic are the local anesthetics (20). In addition, they are metabolized by esterases in the aqueous phase (21). The present results indicate that the pattern of increased potency and toxicity as a function of increased membrane binding is, at least partly, due to the membrane-conferred protection against hydrolysis in the aqueous phase.

Thus, although in the present case the reaction does not take place in the membrane phase , changes in the ESR spectra allow the analysis of the reaction kinetics. This is a novel application of membrane spin probes whereby one can examine how membranes affect basic physico-chemical properties of partitioning compounds.

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