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Interaction of the Local Anesthetics Dibucaine and Tetracaine with Sarcoplasmic Reticulum Membranes. Differential Scanning Calorimetry and Fluorescence Studies†

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Received April 22, 1988; Revised Manuscript Received December 2, 1988

ABSTRACT: The local anesthetics dibucaine and tetracaine inhibit the (Ca^{2+} + Mg^{2+})-ATPase from skeletal muscle sarcoplasmic reticulum [DeBoland, A. R., Jilka, R. L., & Martonosi, A. N. (1975) *J. Biol. Chem. 250*, 7501-7510; Suko, J., Winkler, F., Scharinger, B., & Hellmann, G. (1976) *Biochim. Biophys. Acta 443*, 571-586]. We have carried out differential scanning calorimetry and fluorescence measurements to study the interaction of these drugs with sarcoplasmic reticulum membranes and with purified (Ca^{2+} + Mg^{2+})-ATPase. The temperature range of denaturation of the (Ca^{2+} + Mg^{2+})-ATPase in the sarcoplasmic reticulum membrane, determined from our scanning calorimetry experiments, is ca. 45-55 °C and for the purified enzyme ca. 40-50 °C. Millimolar concentrations of dibucaine and tetracaine, and ethanol at concentrations higher than 1% v/v, lower a few degrees (°C) the denaturation temperature of the (Ca^{2+} + Mg^{2+})-ATPase. Other local anesthetics reported to have no effect on the ATPase activity, such as lidocaine and procaine, did not significantly alter the differential scanning calorimetry pattern of these membranes up to a concentration of 10 mM. The order parameter of the sarcoplasmic reticulum membranes, calculated from measurements of the polarization of the fluorescence of diphenylhexatriene, is not significantly altered at the local anesthetic concentrations that shift the denaturation temperature of the (Ca^{2+} + Mg^{2+})-ATPase. It has been found, however, that the intrinsic fluorescence of these membranes is largely quenched by these local anesthetic concentrations and that this quenching of the intrinsic fluorescence can be adequately fitted to the theoretical energy-transfer prediction using the membrane/water partition coefficients determined in this study. It is suggested that the shift of the denaturation temperature of the (Ca^{2+} + Mg^{2+})-ATPase by these anesthetics, and likely the inhibition of this activity, is related to the progressive disruption of the lipid annulus by these drugs.

A basic question underlying the studies focused on the molecular mechanism of anesthesia is the chemical nature of the anesthetic binding centers. From this perspective, it is to be recalled that previous attempts directed to support the hypothesis that perturbation of the lipid bilayer structure can account for the functional alterations linked to anesthesia have failed so far in providing major structural changes at physiologically relevant concentrations of anesthetics (Franks & Lieb, 1982; Dodson & Moss, 1984). Alternatively, it had been suggested that proteins can provide the binding sites for anesthetics with sufficient specificity and affinity (Franks & Lieb, 1982, 1986; Dodson & Moss, 1984). Because of the relatively high hydrophobic properties of these drugs, these binding sites must be hydrophobic. This and the well-known strong correlation between anesthetic potency and solubility of anesthetics in lipid bilayers suggest the possibility that the interaction of these drugs with hydrophobic binding sites in membrane proteins could be involved in the perturbations of cellular excitability produced at their pharmacological doses. In this regard, it is to be noted that several recent studies have shown

† This work has been supported by Grant 2813/13 from the Spanish CAICYT and by a grant from the Fondo de Investigaciones Sanitarias de la Seguridad Social. A preliminary account of this work was presented at the 9th International Biophysics Congress, Jerusalem, Israel, Aug 1987.

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0006-2960/89/0428-3398$01.50/0

that local anesthetics interact with some membrane proteins (Casanovas et al., 1985; Blanchard et al., 1979; Kresheck et al., 1985) and with calmodulin (an important modulator of cellular excitability) (Tanaka & Hidaka, 1981; Volpi et al., 1981) with relative affinities that correlate well with their relative anesthetic potency. Moreover, Greenberg and Tsong (1982) have demonstrated that membrane proteins provide the local anesthetic receptor site in axonal membranes and, later, that the membrane protein providing such a receptor site is unlikely to be the Na^+ channel (Greenberg & Tsong, 1984).

It has been shown that local anesthetics inhibit the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum, both from skeletal muscle (DeBoland et al., 1975; Suko et al., 1976) and from cardiac muscle (Katz et al., 1975). From kinetic studies, it was concluded that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity by dibucaine and tetracaine correlates well with the decrease of the steady-state phosphoenzyme level (Suko et al., 1976). Kurebayashi et al. (1982) suggested that some of the effects of tetracaine can be rationalized by a specific decrease of the rate of EP decomposition and stabilization of the E_1·ATP·Ca^{2+} complex. However, the detailed molecular mechanism of this inhibition has not been sufficiently clarified. In particular, the putative presence of binding sites in the enzyme whose saturation by local anesthetics leads to inhibition has not been established yet. The alternative hypothesis, namely, that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase could be rationalized in terms of a structural perturbation of the lipid bilayer structure, without the need of direct interaction between the drug and the enzyme, has not been rigorously excluded so far. Because this enzyme is one of the best-known membrane proteins from a biochemical and biophysical point of view, it seems specially suited to gain a deeper knowledge about the functional nature of local anesthetic binding sites in proteins. On the other hand, the properties of these binding sites are likely to be specially relevant for the understanding of modulation of other Ca^{2+} pumps by these drugs, such as the (Ca^{2+} + Mg^{2+})-ATPase of synaptosomal plasma membrane which is inhibited by local anesthetics at concentrations close to their pharmacological levels (Garcia-Martin & Gutiérrez-Merino, 1986).

Differential scanning calorimetry has been shown to be very useful to study ligand–protein interactions (Sturtevant, 1977), lipid–protein interactions (McElhaney, 1986), and conformational shifts of proteins (Biltonen & Freire, 1978). In particular, this technique has proven to be very useful to monitor the interactions of local anesthetics with the F_1-ATPase (Kresheck et al., 1985). Thus, we have used this technique to reveal the existence of interactions of the local anesthetics dibucaine and tetracaine with the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum. The extent of interaction of these local anesthetics with sarcoplasmic reticulum membranes has also been quantified from the extinction of their intrinsic fluorescence by these drugs and by measuring their partition coefficient in our experimental conditions.

Materials and Methods

Sarcoplasmic reticulum has been purified from rabbit (New Zealand White) hind leg muscle as indicated elsewhere (Mata & Gutiérrez-Merino, 1985). (Ca^{2+} + Mg^{2+})-ATPase was purified from sarcoplasmic reticulum following the procedure of MacLennan (1970) and by affinity chromatography through Affi-Gel Blue as described by Gafni and Boyer (1984). The purity of the (Ca^{2+} + Mg^{2+})-ATPase was checked by SDS gel electrophoresis (7.5% acrylamide) and found to be more than 95% pure from the absorbance at 550 nm of Coomassie Blue stained gels. Protein concentration was measured following the method of Lowry et al. (1951), using bovine serum albumin as standard. Lipids were determined as total phosphorus following the method of Barlett (1964). The (Ca^{2+} + Mg^{2+})-ATPase activity has been measured by using the coupled enzyme system (Warren et al., 1974) with the following assay mixture: 0.1 M *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (Tes)[1]/0.1 M KCl/5 mM $MgCl_2$/0.1 mM $CaCl_2$/2.5 mM ATP/0.2 mM NADH/0.42 mM PEP/7.5 IU of pyruvate kinase/18 IU of lactate dehydrogenase (pH 7.45). The effects of local anesthetics on the (Ca^{2+} + Mg^{2+})-ATPase activity were further assessed by direct measurements of the released inorganic phosphate following the method of Fiske and Subbarow (Leloir & Cardini, 1957). The Ca^{2+}-independent ATPase activity was measured in the presence of 3.4 mM EGTA, and only those preparations showing a value of this activity lower than 10% of total uncoupled (Ca^{2+} + Mg^{2+})-ATPase activity were used in this study. "Leakiness" of sarcoplasmic vesicles was assessed by measuring the (Ca^{2+} + Mg^{2+})-ATPase activity in the presence and in the absence of A23187 (1–2 μg/mL). Only those preparations showing a 3–4-fold stimulation of the (Ca^{2+} + Mg^{2+})-ATPase activity at 20–22 °C upon addition of A23187 have been used in this study. The specific activity of purified (Ca^{2+} + Mg^{2+})-ATPase ranged typically between 5 and 7 IU at 25 °C and contained 35–40 mol of lipid/mol of protein monomer.

Scanning calorimetry measurements have been carried out by using a Microcal MC-2 differential scanning calorimeter operating at a scanning rate of 30 °C/h under ca. 1.5 kg/cm^2 pressure during the scan. The buffer used in scanning calorimetry experiments has been 50 mM Tes/0.1 M KCl/0.25 M sucrose/2 mM dithiothreitol (pH 7.4). The samples were carefully degasified before the calorimeter was loaded. The pH was carefully readjusted to 7.4 when necessary after addition of the local anesthetic. Fluorescence measurements have been carried out in buffer using a Hitachi Perkin-Elmer spectrofluorometer, Model 650-40, operating in the ratio mode at 27–37 °C with the excitation wavelength at 280 nm. The intrinsic fluorescence of SR membranes was found to continuously decay as a function of time, but after 25–30 min, this process is sufficiently slow to allow reliable titration experiments of this fluorescence with increasing concentrations of the local anesthetics. This has also been observed by other investigators (Guillain et al., 1980; Froud & Lee, 1986). Under our experimental conditions, in the 20–25 min after diluting a few microliters of SR membranes stored at 4 °C in a solution preequilibrated at 25 °C, the decay of the intrinsic fluorescence intensity is 20–25% of its initial value, i.e., at an average rate of ca. 1% intensity decay per minute. It has been suggested that this quenching of the intrinsic fluorescence can be due to slow denaturation of the (Ca^{2+} + Mg^{2+})-ATPase (Froud & Lee, 1986), possibly caused by photolytic decomposition of Tyr and Trp.

As illustrated later under Results, the emission spectra of tetracaine and dibucaine partially overlap with the emission spectra of the intrinsic fluorescence of SR membranes. To adequately estimate the extent of intrinsic fluorescence quenching at a given concentration of the local anesthetics, thus, the emission spectra have been deconvoluted in the sum

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IU, micromoles of product per minute per milligram of protein; PEP, phosphoenolpyruvate; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

of two bands centered at the emission maximum wavelengths of the intrinsic fluorescence and of the local anesthetic. Each band has been fitted by using skewed Gaussians, as shown by Rooney and Lee (1986), and the proper parameters determined from the best least-squares fit of the emission spectra of each fluorophore determined independently. The fit of the emission spectra of a solution containing the local anesthetic and SR membrane has been carried out by computation, allowing the intensity of the two peaks to vary to match the recorded spectra. This approach rests on the assumption that the dibucaine and tetracaine emission spectra are little affected upon adsorption to the membrane. Previous studies have shown that adsorption of these local anesthetics on lipid bilayers produces only a slight shift ($\leq$5 nm) of their maximum emission wavelength (Guilmin et al., 1982). The error bars in the estimated extent of quenching of the intrinsic fluorescence by these local anesthetics given under Results (Figure 6) reflect the effect of this shift on the calculated quenching.

The measurements of the polarization of fluorescence of DPH have been carried out at 25 °C with excitation and emission wavelengths of 360 and 440 nm, respectively. Polarization of fluorescence, $P(\lambda)$, was calculated by using the expression (Lee, 1982):

$$P(\lambda) = \frac{I_{\parallel}(\lambda) - GI_{\perp}(\lambda)}{I_{\parallel}(\lambda) + GI_{\perp}(\lambda)}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities measured with parallel and perpendicularly oriented polarizers, respectively, and G is the correction factor for polarization characteristics of the emission monochromator. A value of 1.04 ± 0.02, averaged from more than 30 measurements, has been used in our calculations. The steady-state anisotropy of fluorescence, r_s, has been calculated from polarization (P) by using the equation (Lee, 1982):

$$r_s = 2P/(3 - P)$$

Analysis of Fluorescence Energy Transfer. Fluorescence energy transfer between protein and local anesthetics adsorbed in the SR membrane has been analyzed following the theoretical approach outlined in Gutiérrez-Merino (1981) and Gutiérrez-Merino et al. (1987). Briefly, in the presence of acceptors, the fluorescence of the donor can be expressed as

$$(F_0 - F)/F_0 = \langle E \rangle = (\langle k \rangle/k_0)/(1 + \langle k \rangle/k_0) \quad (1)$$

where F_0 and F are fluorescence intensities for the donor in the absence and presence of acceptor, respectively, $\langle k \rangle$ is the average rate of Forster energy transfer for the particular distribution of acceptors, and k_0 is the rate of energy transfer for a donor–acceptor pair separated by the distance R_0 at which the efficiency of energy transfer, $\langle E \rangle$, is 50%.

The average rate of energy transfer $\langle k \rangle$ in a two-dimensional membrane system can be written as

$$\langle k \rangle = \sum_{i=1}^{n} k_i \quad (2)$$

where the summation extends over all donor–acceptor pairs of the ensemble and where the rate of transfer for pair i separated by a distance r_i is k_i, provided that the diffusion rate of donor and acceptor is much slower than the lifetime of the excited state of the donor. For the computation of the value of $\langle k \rangle$, we have assumed a random distribution of the local anesthetic on the lipid bilayer, with no specific interaction with the protein. Considering the structural data available (Kuroda & Fujiwara, 1987) and the saturation characteristics of the binding of local anesthetics to lipid bilayers (Ohki, 1984; Eftink et al., 1985), the positions of the local anesthetics have been fixed for computation in the center of the maximum packed hexagonal lattice of lipids. Within this framework, the optimal fit of available energy-transfer data in mixed lipid bilayers (donor lipid–acceptor lipid), such as the data of Fung and Stryer (1978) and Gutiérrez-Merino et al. (1987), is obtained by using and average lipid cross-sectional diameter of 7.5 Å at the lipid–water interface. This value is not far from the value that can be derived from structural studies of lipid molecules in native biological membranes (Huang & Mason, 1978).

The average position of donor Trp in the (Ca^{2+} + Mg^{2+})-ATPase with respect to the lipid–water interface determines the distance of closest approach to acceptor anesthetics in the bilayer. This position has been characterized in terms of the perpendicular distance to the plane of the lipid–water interface (h) and the distance to the lipid–protein interface in a cross section parallel to the lipid–water interface (d), as illustrated in detail in Gutiérrez-Merino et al. (1987).

Chemicals. Bovine serum albumin, deoxycholate, DPH, ATP, phosphoenolpyruvate, EGTA, dibucaine, procaine, lidocaine, tetracaine, phenylmethanesulfonyl fluoride, β-mercaptoethanol, imidazole, and Tes were obtained from Sigma. A23187, pyruvate kinase, and lactate dehydrogenase were purchased from Boehringer Mannheim. All the other chemicals used in this study were obtained from Merck. Affi-Gel Blue (100–200 mesh) was obtained from Bio-Rad. Triton X-100 is a trademark of Rohm & Haas, Co. Tetracaine solutions were freshly prepared before use.

Results

Partition Coefficients of Local Anesthetics on Sarcoplasmic Reticulum Membranes. The local anesthetics used in this study (dibucaine, tetracaine, lidocaine, and procaine) are hydrophobic compounds whose pK in aqueous solution ranges between 8.0 and 8.9 (Ohki, 1984). Therefore, at pH 7.4–7.5, the pH of the buffers used in our study, they exist in two forms in equilibrium: neutral and cationic. In order to properly rationalize the effects produced by these drugs in sarcoplasmic reticulum membranes, we need to know to what extent these anesthetics are bound to the membranes and, thus, their respective aqueous and membrane concentrations.

The partition coefficients of these drugs between water and sarcoplasmic reticulum membranes have been calculated spectrophotometrically by measuring the aqueous local anesthetic concentration after pelleting the membranes, at several membrane concentrations ranging from 0 up to 6.5 mg of protein/mL. To achieve this, sarcoplasmic reticulum membranes have been centrifuged for 30 min at an average of 100000g, after a preincubation of 30 min at room temperature with a given concentration of the local anesthetic. The aqueous concentrations of the anesthetics have been calculated from absorbance measurements of the supernatant at the appropriated wavelengths. For the local anesthetics used here, their absorbance peak wavelengths, determined from their absorbance spectra, were as follows: dibucaine, 328 nm; tetracaine, 310 nm; procaine, 295 nm; lidocaine, 273 nm. In order to minimize errors in the determination of the ratio between membrane-bound and aqueous local anesthetic concentrations (K_p), the total concentration of each anesthetic was also determined spectrophotometrically in the cuvettes before adding sarcoplasmic reticulum membranes. From these measurements, the following apparent K_p values have been obtained: 0.75 (dibucaine), 0.81 (tetracaine), and less than 0.1 for lidocaine and procaine. It is to be noted that at high local anesthetic concentrations the apparent value of the partition

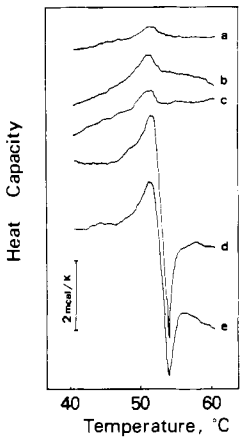

FIGURE 1: Calorimetric scans of sarcoplasmic reticulum membranes. From a to e, the membrane concentrations were 0.5, 0.8, 1.0, 1.4, and 2.0 mg of protein/mL. The recorder traces have been shifted to avoid overlap of signals. Scanning rate, 30 °C/h; buffer, 50 mM Tes/0.1 M KCl/0.25 M sucrose/2 mM dithioithreitol (pH 7.4). Other experimental conditions as indicated under Materials and Methods.

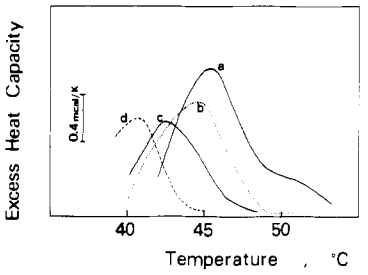

FIGURE 2: Calorimetric scans of purified (Ca^{2+} + Mg^{2+})-ATPase. Protein concentration, 2 mg/mL. The different scans shown correspond to the following experimental conditions: (a) protein in buffer; (b) protein in the presence of 2 mM dibucaine; (c and d) protein in the presence of 3 and 4 mM tetracaine, respectively. Buffer and other experimental conditions are the same as those of Figure 1.

coefficient decreases. This is likely to reflect the saturation of the lipid bilayer by the local anesthetic, a phenomenon already described for pure lipid bilayers (Ohki, 1984; Eftink et al., 1985). In our experimental conditions, the value of K_p is constant up to total local anesthetic concentrations of ca. 1 mM at a protein concentration of 6.5 mg/mL. It appears from these data that adsorption of these anesthetics on the sarcoplasmic reticulum membrane is much weaker than their adsorption on pure lipid systems. The high density of protein particles in the sarcoplasmic reticulum membrane, which protrudes from the lipid–water interface of 40–60 Å (Taylor et al., 1986), is likely to decrease the accessibility of sarcoplasmic reticulum membrane lipids to local anesthetics and, thus, decrease their partition coefficient with respect to that of pure lipid–water systems.

In addition, because it has been shown that large concentrations of tetracaine (≥50 mM) produce the formation of mixed micelles with phosphatidylcholine and disrupt the lipid bilayer (Fernández, 1981), we have studied the effects of these local anesthetics at the concentrations used in this study on the integrity of the sarcoplasmic reticulum membrane, e.g., up to 10 mM procaine, up to 10 mM lidocaine, up to 4 mM tetracaine, and up to 2 mM dibucaine. None of the local anesthetics studied here induce a significant micellization of these membranes in our experimental conditions, as indicated by the negligible effect of these drugs on the turbidity of sarcoplasmic reticulum membrane suspensions measured at 400 nm, except for the case of dibucaine where the measurements were carried out at a larger wavelength in order to minimize the absorption of this anesthetic (results not shown). This was further tested by measuring the effects of these local anesthetic concentrations on the permeability of sarcoplasmic reticulum membranes to sucrose by the osmotic shock technique, as in Escudero and Gutiérrez-Merino (1987). None of these local anesthetics in the concentration range referred to above did significantly alter the rate of sucrose influx across these membranes (half-time of ca. 120 min) after an osmotic shock with 250 mM sucrose [results not shown; see also Escudero and Gutiérrez-Merino (1987)].

Differential Scanning Calorimetry Studies. The results of typical scans of sarcoplasmic reticulum membranes are presented in Figure 1 at several membrane concentrations. The results given in the figure are representative of those obtained with, at least, five preparations of sarcoplasmic reticulum membranes. It can be observed that there is an endothermic peak centered at ca. 51–52 °C, which consistently increases as the protein concentration increases. Because protein denaturation is an endothermic process (Sturtevant, 1977; Biltonen & Freire, 1978) and also because membrane proteins have been reported to undergo denaturation in this temperature range (Davio & Low, 1982; Farach & Martinez-Carrión, 1983; McElhaney, 1986), it is likely that this peak corresponds to the denaturation of the sarcoplasmic reticulum membrane proteins. (Ca^{2+} + Mg^{2+})-ATPase is the major protein component of these membranes, amounting from 70 to 90% of the total sarcoplasmic reticulum protein (Tada et al., 1978; Møller et al., 1982). In addition, Jaworsky et al. (1986), using Fourier-transform infrared spectroscopy, have shown that the (Ca^{2+} + Mg^{2+})-ATPase from sarcoplasmic reticulum denatures between 40 and 50 °C, the process being correlated with a large change of the α-helix content of this protein. Partial heating of these samples up to ca. 50–55 °C and then cooling the samples produce a flat reheating scan that overlaps the base line, thus showing that the denaturation of sarcoplasmic reticulum membranes is an irreversible process. To further assess this point, we have purified the (Ca^{2+} + Mg^{2+})-ATPase as indicated under Materials and Methods, and the heating of the samples gave results such as those presented in the Figure 2, where data representative from those obtained with three different preparations are shown. Similar results were obtained using (Ca^{2+} + Mg^{2+})-ATPase purified by affinity chromatography using Affi-Gel Blue, following the method developed by Gafni and Boyer (1984) (results not shown). The agreement between the temperature range for denaturation of the (Ca^{2+} + Mg^{2+})-ATPase estimated from infrared spectroscopy and from scanning calorimetry of the purified enzyme is, thus, excellent. A break in the temperature dependence of the intrinsic fluorescence of sarcoplasmic reticulum membranes and in the fluorescence of fluorescein, covalently attached to the catalytic center of the (Ca^{2+} + Mg^{2+})-ATPase following a protocol previously described (Mata & Gutiérrez-Merino, 1985), has been also found in this temperature range (results not shown). These results, in turn, show that the active conformation of the (Ca^{2+} + Mg^{2+})-ATPase is different in native sarcoplasmic reticulum membranes and in purified (Ca^{2+} + Mg^{2+})-ATPase preparations.

At higher protein concentrations, e.g., above 1 mg/mL, a second peak, exothermic, is observed in the scans of sarcoplasmic reticulum membranes (Figure 1), but not in those of the purified (Ca^{2+} + Mg^{2+})-ATPase (Figure 2). Because unspecific aggregation of membranes is largely exothermic (Privalov & Khechinashvili, 1974), it seems likely that this peak corresponds to a strong aggregation of these membranes induced by their denaturation. This aggregation is clearly seen upon direct inspection of highly concentrated samples of

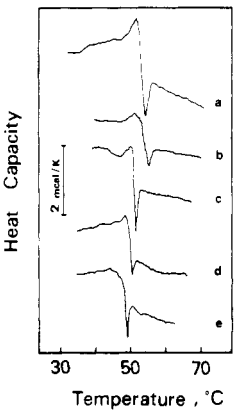

FIGURE 3: Effect of dibucaine on the calorimetric scans of sarcoplasmic reticulum membranes. Protein concentration, 2 mg/mL. From a to e, the total concentrations of dibucaine were 0, 0.5, 1, 1.5, and 2 mM. Buffer and other experimental conditions are as indicated in Figure 1.

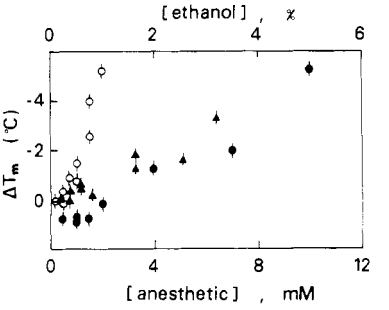

FIGURE 4: Dependence of the temperature of denaturation of sarcoplasmic reticulum membranes (T_m) upon the total concentration of the local anesthetic dibucaine (○), tetracaine (●), and ethanol (▲). Data obtained from calorimetric scans like those shown in Figure 3. The shift of the temperature of denaturation (ΔT_m) has been estimated from the beginning of the steep decrease of the heat capacity in the denaturation range. Similar results can be obtained when plotting the sharp minimum, corresponding to the strong exothermic peak after denaturation. The error bars are the estimated average error of T_m as determined from duplicate or triplicate experiments in the same experimental conditions.

sarcoplasmic reticulum membranes, and it is expected to be strongly dependent upon protein concentration. Furthermore, these membranes show a strong tendency to interact and aggregate above a concentration of 1 mg of protein/mL, as indicated by an altered–SH reactivity pattern (Georgoussi & Stiroudis, 1985) and laser scattering measurements (Arrio et al., 1974). We have also found that this exothermic peak is present in scans carried out in the presence of high concentrations of thiol-protecting reagents, such as 40 mM DTT or β-mercaptoethanol, therefore indicating that even if disulfide bridges are extensively formed in this process, these are not the major determinant of it. On the other hand, the presence of this large peak in sarcoplasmic reticulum membranes makes unrealistic the accurate estimation of the enthalpy change of the endothermic peak. For the case of purified (Ca^{2+} + Mg^{2+})-ATPase, a value of 450 ± 50 kcal/mol of monomer, e.g., 3.9 ± 0.4 cal/g of protein, can be derived from the scans, a value which is in good agreement with the literature value reported for other membrane proteins of large molecular weight (Kresheck et al., 1985; Yu et al., 1985; McElhaney, 1986). This enthalpy change is likely to include any enthalpic contribution due to the rapid aggregation between protein monomers in a vesicle that should follow the denaturation process.

We have studied the effect of the local anesthetics dibucaine, tetracaine, procaine, and lidocaine on the denaturation of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum. Because of the low solubility of these local anesthetics, they have been added from an ethanolic solution to reach the higher concentrations of some of them, namely, lidocaine, dibucaine, and tetracaine. Therefore, we have also studied the effect of ethanol (a local anesthetic as well) on the denaturation of these membranes. Ethanol up to concentrations as high as 1% v/v does not significantly alter either the peak height (not shown) or the transition temperature (Figure 4). On these grounds, care has been taken to add less than 1% ethanol when it has been necessary to add the local anesthetic from an ethanolic solution.

Dibucaine and tetracaine clearly shift the temperature of denaturation toward lower temperatures (Figures 3 and 4). However, up to 10 mM, neither procaine nor lidocaine, reported to be ineffective on the ATPase activity in this concentration range (DeBoland et al., 1975; Suko et al., 1976), significantly alters the denaturation profile of sarcoplasmic reticulum membranes. Taking into account the complex nature of the denaturation peak in calorimetric scans (see above), it is likely that the slight raise of T_m by low tetracaine concentrations is attributable to an altered position of the exothermic peak. In addition, these local anesthetics appear to decrease the area of the endothermic peak. This effect is more readily seen when the calorimetric scan of the purified (Ca^{2+} + Mg^{2+})-ATPase is carried out in the presence of tetracaine or dibucaine (Figure 2). In this latter case, the shift of the denaturation temperature is clearly lower than the shift induced by the same concentrations of these local anesthetics in the sarcoplasmic reticulum membrane (compare Figures 2 and 4). Because of the strong adsorption of these local anesthetics to sarcoplasmic reticulum membranes, at a concentration of 2 mg of protein/mL, the free concentrations of these drugs have to be corrected by using their partition coefficients (see above). This correction cannot be used to explain the above-mentioned differences, for the aqueous concentration of the local anesthetic at a given total local anesthetic concentration should be lower in the experiments involving sarcoplasmic reticulum membranes than in those carried out with purified (Ca^{2+} + Mg^{2+})-ATPase, due to the higher lipid content of the native membranes (see Materials and Methods). Nevertheless, it is to be noted that the endothermic peak of the sarcoplasmic reticulum melting profile is shifted by local anesthetics about the same magnitude as it is in the purified (Ca^{2+} + Mg^{2+})-ATPase in the absence of anesthetic with respect to native sarcoplasmic reticulum membranes.

Quenching of the Intrinsic Fluorescence of Sarcoplasmic Reticulum by Local Anesthetics. Nearly 95% of total Tyr and Trp of sarcoplasmic reticulum membranes belong to the (Ca^{2+} + Mg^{2+})-ATPase (Møller et al., 1982). The effects of tetracaine and dibucaine upon the intrinsic fluorescence of sarcoplasmic reticulum have been studied (Figure 5). Because of the low local anesthetic concentrations used in these experiments, the anesthetics were added from stock aqueous solutions. The extent of quenching was found to be unaffected by temperature in the range of 27–37 °C. Both local anesthetics quench the intrinsic fluorescence of these membranes. The overlap between the emission band of the intrinsic fluorescence and the absorption spectra of both dibucaine and tetracaine strongly suggests that this quenching can be due to fluorescence energy transfer between Trp and these local anesthetics. The quenching by the local anesthetics is fully reverted upon elimination of the drug by dialysis of the samples against buffer for 12 h at 4 °C (results not shown). This supports the view that the interaction of these drugs with

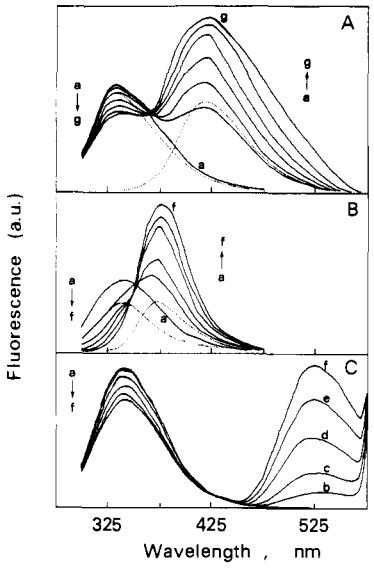

FIGURE 5: Quenching of the intrinsic fluorescence of sarcoplasmic reticulum membranes (λ_{exc} = 280 nm) by the local anesthetics dibucaine (panel A) and tetracaine (panel B) and the analogue quinacrine (panel C). Protein concentration, 50 μg/mL. (Panel A) From a to g, the total concentrations of dibucaine were 0, 5, 12, 15, and 20 μM. (Panel B) From a to f, the total concentrations of tetracaine were 0, 10, 20, 30, 40, and 50 μM. (Panel C) From a to f, the total concentrations of quinacrine were 0, 0.5, 1, 2, 3, and 4 μM. Buffer, 50 mM Tes/0.1 M KCl/0.25 M sucrose/0.1 mM phenylmethanesulfonyl fluoride/0.2 mM β-mercaptoethanol (pH 7.4). The results presented in the figure are a typical experimental series obtained with one sarcoplasmic reticulum preparation. Similar results have been obtained with, at least, three different preparations. The dotted lines in panels A and B represent the results of deconvolution of spectrum c of panel A and of spectrum b of panel B and are included to illustrate this point.

sarcoplasmic reticulum membranes can be treated as a reversible process, i.e., that protein denaturation is unlikely to be the cause of the quenching of Trp fluorescence by these drugs. Because these local anesthetics also exhibit fluorescence with emission peaks not largely separated from that of the intrinsic fluorescence emission band, to quantitate the extent of fluorescence quenching we have used a computer-based approach for deconvolution of the two emission bands, i.e., that of the local anesthetic and that of the intrinsic fluorescence. These bands have been simulated by means of skewed Gaussians as indicated under Materials and Methods. The calculated extent of quenching is presented in the Figure 6 as a function of the surface density of local anesthetic. We have also studied the local anesthetic analogue quinacrine (Greenberg & Tsong, 1982), in order to carry out the analysis of the quenching of the intrinsic fluorescence of sarcoplasmic reticulum membranes with a compound of hydrophobicity similar to that of dibucaine (calculated K_p value of 1.2 ± 0.2 at 6 mg of protein/mL), but showing an emission band clearly separated from that of the intrinsic fluorescence. The results obtained are presented in Figure 5 and plotted as the extent of quenching versus bound drug concentration in Figure 6.

The values of R_0 for energy transfer between the intrinsic fluorescence of sarcoplasmic reticulum and these local anesthetics have been calculated from the absorption spectra of local anesthetics and corrected intrinsic fluorescence emission spectra, as indicated elsewhere (Gutiérrez-Merino et al., 1987). The relevant spectroscopy constants are presented in Table I. The quantum yield of the intrinsic fluorescence of sarcoplasmic reticulum was taken as 0.11 ± 0.03, averaged from the value of this parameter in proteins (Lakowicz, 1983). To estimate the overlap integral, we have used a wavelength interval of 5 nm, and the refractive index, n, was taken as 1.33, i.e., that of dilute aqueous solutions, as in Gutiérrez-Merino et al. (1987). The continuous lines on Figure 6 have been calculated by using the values of R_0 given in the Table I, the molar fraction of these local anesthetics in sarcoplasmic reticulum membranes determined from the apparent partition constants given above, and setting the closest approach distance between donor and acceptors indicated in the legend of this Figure. It is to be noted that this closest approach distance is ca. 5 Å shorter for the case of tetracaine.

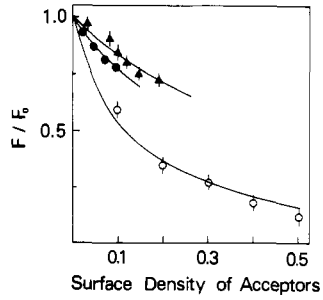

FIGURE 6: Extent of quenching of the intrinsic fluorescence of sarcoplasmic reticulum membranes versus the surface density of local anesthetic. The symbols correspond to dibucaine (▲), quinacrine (●), and tetracaine (○). The extent of quenching has been expressed as the fluorescence in the presence of the local anesthetic (F) over the fluorescence in the absence of the anesthetic (F_0), and it has been calculated from data like those presented in Figure 5 as explained in the text. The surface density has been expressed as the ratio between the concentration of local anesthetic bound to the sarcoplasmic reticulum membrane and the total lipid concentration in the cuvette. The concentration of the local anesthetic bound to the membrane has been estimated by using the apparent adsorption coefficients given in the text. The solid lines represent the best fit of the experimental data to quenching by energy transfer, using the R_0 values given in Table I and the following distances of closest approach (d, h, in angstroms): dibucaine and quinacrine, (5,17) or (7,15); tetracaine, (7,10) or (5,12).

Table I: Energy Transfer between the Intrinsic Fluorescence of Sarcoplasmic Reticulum (SR) and Local Anesthetics[a]

	ϵ^b ($M^{-1}\cdot cm^{-1}$)	J ($cm^3\cdot M^{-1}$)	R_0 (Å)[c]
SR–dibucaine	6000	0.280×10^{-14}	18.2
SR–tetracaine	23000	0.803×10^{-14}	21.7
SR–quinacrine	4100	0.515×10^{-14}	20.1

[a] Relevant spectral parameters. [b] At the peak wavelength of their respective bands in the 280–360-nm range: 328 nm (dibucaine); 310 nm (tetracaine), and 344 nm (quinacrine). [c] Obtained by using the following values of the parameters indicated: refractive index = 1.33; $K^2 = {}^2/_3$; quantum yield of the donor in the absence of acceptor = 0.11 (see the text for further explanations).

Discussion

The calorimetric results presented in this paper indicate that both local anesthetics, as well as ethanol, shift the (Ca^{2+} + Mg^{2+})-ATPase to a more unstable conformational state in native membranes at concentrations close to those that inhibit the (Ca^{2+} + Mg^{2+})-ATPase activity. This effect is also observed with purified (Ca^{2+} + Mg^{2+})-ATPase, although in this latter case with much lower intensity (compare Figures 2–4), probably because the purification of this enzyme leads to a conformation of the (Ca^{2+} + Mg^{2+})-ATPase similar to that favored by the above-mentioned local anesthetics. This hypothesis is supported by the strong shift of the denaturation peak of the (Ca^{2+} + Mg^{2+})-ATPase toward lower temperatures upon purification. In addition, these results suggest that the effect of dibucaine and tetracaine upon the stability of the (Ca^{2+} + Mg^{2+})-ATPase is related to the disruption of the lipid annulus surrounding the enzyme in native membranes.

The possibility of interaction of these local anesthetics with specific binding sites in the protein structure deserves discussion. Kinetic studies have shown that at the concentrations that inhibit this enzyme there is only a small effect of dibucaine and tetracaine on the Ca^{2+} dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity (Suko et al., 1976; results not shown) and that there is no kinetic competition between ATP and these local anesthetics and between Mg^{2+} and these drugs (results not shown). Therefore, it can be excluded that the inhibition by dibucaine and tetracaine results from binding to Ca^{2+} or Mg^{2+} or ATP sites in the ATPase. The existence of nonannular binding sites for hydrophobic drugs in the (Ca^{2+} + Mg^{2+})-ATPase has been shown. Nevertheless, it is to be recalled that these hydrophobic sites have been suggested to be located at the protein–protein interface within an oligomer, e.g., at the protein surface that results exposed to the lipid upon dissociation of the oligomer into monomers (Simmonds et al., 1982). The hypothesis of displacement of lipids upon binding of these anesthetics to annular sites is consistent with the major structural alterations induced by delipidation of the (Ca^{2+} + Mg^{2+})-ATPase by detergents (Tada et al., 1978; The et al., 1981; Møller et al., 1982), structural changes that lead to an irreversible denaturation of this enzyme system, with the fact th it procaine and lidocaine, which show a much lower partition coefficient in sarcoplasmic reticulum membranes (see above), do not exert any significant perturbation of the calorimetric scans. In addition, this hypothesis is also consistent with the fact that the sensitivity of purified (Ca^{2+} + Mg^{2+})-ATPase to inhibition by dibucaine and tetracaine ($K_{0.5}$ values of 0.25 ± 0.05 and 0.9 ± 0.1 mM, respectively; results not shown) is clearly higher than that of sarcoplasmic reticulum membranes of larger lipid to protein ratio ($K_{0.5}$ values of 1.1 ± 0.1 and 3.2 ± 0.3 mM, respectively) (Escudero & Gutiérrez-Merino, 1987). The need of annular lipids to support an efficient activity of this enzyme has been well established (London & Feigenson, 1981; Caffrey & Feigenson, 1981; East & Lee, 1982), whereas saturation of nonannular hydrophobic binding sites can even activate this enzyme (Simmonds et al., 1982; Jones & Lee, 1985). The very abrupt dependence of the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase upon dibucaine and tetracaine concentration (showing apparent Hill coefficients of ca. 4–5) also supports the hypothesis that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity by these local anesthetics is directly related to the disruption of the lipid annulus around the protein, because it has been shown that the activity of intrinsic membrane proteins shows allosteric behavior with respect to the saturation of their annular binding sites by lipids (Sandermann, 1983).

The quenching of the intrinsic fluorescence of the sarcoplasmic reticulum membrane by dibucaine and tetracaine can be adequately fitted by Forster energy-transfer theoretical predictions, using the partition coefficients for these drugs determined in this study (solid lines in the Figure 6). By itself, this result indicates a homogeneous distribution of the local anesthetic in the sarcoplasmic reticulum lipid bilayer. Therefore, the lipid annulus around the (Ca^{2+} + Mg^{2+})-ATPase should be progressively saturated by the anesthetic as its concentration in the membrane increases. In addition, from the theoretical analysis of these results, it is concluded that the local anesthetics in the bilayer locate ca. 12–18 Å away from the core of Trp residues on the (Ca^{2+} + Mg^{2+})-ATPase. It is to be noted that for the case of tetracaine the closest approach distance has been estimated to be about 5 Å shorter than that of dibucaine (see the legend of Figure 6), and it has been shown that this local anesthetic penetrates more deeply within the lipid bilayer, e.g., about 4–5 additional $-CH_2-$ groups (Kuroda & Fujiwara, 1987). By contrast, tetracaine shows a weaker effect on the denaturation temperature than dibucaine. However, it is to be noted that dibucaine has a larger effect on the lipid structure than tetracaine, as it is supported by the differential effects of both anesthetics on structural parameters, sodium permeability, and the melting profile of pure lipid monolayers and bilayers (Papahadjopoulos, 1972; Singer & Jain, 1980; results not shown). Procaine also induces a large quenching of the intrinsic fluorescence of the sarcoplasmic reticulum membrane in the concentration range 10–100 μM (results not shown). Thus, the lack of effect of procaine (up to 10 mM) on the differential scanning calorimetry denaturation profile of these membranes cannot be attributed to a negligible adsorption of this drug to the membrane. Rather, it seems that the ability of procaine to disorganize the lipid annulus surrounding the (Ca^{2+} + Mg^{2+})-ATPase is much lower than that of the more hydrophobic local anesthetics, dibucaine and tetracaine.

We have also considered the possibility that the alteration of the (Ca^{2+} + Mg^{2+})-ATPase structure could be caused by membrane fluidity changes. Recent NMR studies (Kuroda & Fujiwara, 1987) have shown that the local anesthetics studied herein do not deeply penetrate within the lipid bilayer, the perturbation of the lipid structure produced by these local anesthetics being mostly centered to the polar headgroup region and the glycerol backbone. Our measurements of the polarization of the fluorescence of DPH fully support that in sarcoplasmic reticulum membranes the basic features of the interaction of local anesthetics with lipids are likely to be similar to those found in lipid bilayers, because there is a negligible effect of these drugs (dibucaine, up to 1 mM; tetracaine, up to 3 mM; ethanol, up to 7% v/v) upon the anisotropy of fluorescence of DPH in these membranes, which was found to be 0.176 ± 0.006 at 25 °C (averaged from more than 10 measurements; results not shown), in good agreement with literature data [reviewed by Pottel et al. (1983)].

Upon adsorption of dibucaine and tetracaine on the lipid bilayer, changes on the surface potential and on the thickness of the sarcoplasmic reticulum membrane are expected to occur. Let us briefly discuss the putative implication of these changes in modulating the (Ca^{2+} + Mg^{2+})-ATPase activity. The catalytic center of the (Ca^{2+} + Mg^{2+})-ATPase is located far from the lipid–water interface (Gutiérrez-Merino et al., 1987; Teruel & Gómez-Fernández, 1986). Thus, the surface potential built up at the lipid–water interface by local anesthetic adsorption into the sarcoplasmic reticulum membrane seems a priori unlikely to significantly contribute to the inhibition of this enzyme by dibucaine and tetracaine. Because of its importance, this point has been analyzed in more detail. Ohki (1984) has reported microelectrophoretic measurements of the ζ potential of egg lecithin liposomes in the presence of dibucaine and tetracaine. At the concentrations of these local anesthetics that produce a significant inhibition of the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum membranes, the induced change of the ζ potential is ca. 20–25 mV. Considering that the partition coefficient of these drugs in this membrane is lower than in egg lecithin bilayers (see above), the actual surface potential change induced by dibucaine and tetracaine will certainly be smaller than that value. The change of potential at the catalytic center due to the adsorption of dibucaine and tetracaine at the concentrations that inhibit the (Ca^{2+} + Mg^{2+})-ATPase has been estimated to be less than 1 mV, using eq 17 of Rooney and Lee (1983) and taking the ζ potential as the potential at ca. 2 Å from the surface of the

membrane (Eisenberg et al., 1979). On the other hand, it has been shown that the (Ca^{2+} + Mg^{2+})-ATPase activity is only slightly dependent on the membrane potential between −50 and 100 mV (Navarro & Essig, 1984).

The thickness of the lipid bilayer has been shown to modulate the activity of the (Ca^{2+} + Mg^{2+})-ATPase using reconstituted membranes (Caffrey & Feigenson, 1981). Changes in the thickness of the bilayer induced by anesthetics have been reported (Franks & Lieb, 1982; Dodson & Moss, 1984). However, these changes are small, of less than 5% (Roth, 1979; Franks & Lieb, 1982; Dodson & Moss, 1984), and the dependence of the (Ca^{2+} + Mg^{2+})-ATPase activity on the thickness of the lipid bilayer is not very steep (Caffrey & Feigenson, 1981).

In conclusion, it is suggested that the inhibition of the (Ca^{2+} + Mg^{2+})-ATPase activity of sarcoplasmic reticulum membranes by dibucaine and tetracaine is due to the disruption of the lipid annulus around this protein. Furthermore, it appears that the lipid–protein interactions in the protein domain in close contact with the lipid and near the lipid–water interface play an important role in determining the conformation of the (Ca^{2+} + Mg^{2+})-ATPase.

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pH-Dependent Bilayer Destabilization and Fusion of Phospholipidic Large Unilamellar Vesicles Induced by Diphtheria Toxin and Its Fragments A and B†

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Received July 20, 1988; Revised Manuscript Received December 6, 1988

ABSTRACT: The passage by the low endosomal pH is believed to be an essential step of the diphtheria toxin (DT) intoxication process in vivo. Several studies have suggested that this low pH triggers the insertion of DT into the membrane. We demonstrate here that its insertion into large unilamellar vesicles (LUV) is accompanied by a strong destabilization of the vesicles at low pH. The destabilization has been studied by following the release of a fluorescent dye (calcein) encapsulated in the liposomes. The influence of the lipid composition upon this process has been examined. At a given pH, the calcein release is always faster for a negatively charged (asolectin) than for a zwitterionic (egg PC) system. Moreover, the transition pH, which is the pH at which the toxin-induced release becomes significant, is shifted upward for the asolectin LUV as compared to the egg PC LUV. No calcein release is observed for rigid phospholipid vesicles (DPPC and DPPC/DPPA 9/1 mol/mol) below their transition temperature whereas DT induces an important release of the dye in the temperature range corresponding to the phase transition. The transition pH associated to the calcein release from egg PC vesicles is identical with that corresponding to the exposure of the DT hydrophobic domains, as revealed here by the binding of a hydrophobic probe (ANS) to the toxin. This suggests the involvement of these domains in the destabilization process. Both A and B fragments destabilize asolectin and PC vesicles in a pH-dependent manner but to a lesser extent than the entire toxin. Phospholipid vesicle fusion mediated by DT or its fragments was monitored in order to evaluate the possible contribution of this process to the release of the liposome internal content. DT and DTB induced fusion of negatively charged vesicles below pH 5 whereas DTA did not fuse any vesicles whatever the composition tested. The role of DTB peptide domains in the fusion and aggregation processes is discussed.

Diphtheria toxin (DT),[1] produced by *Corynebacterium diphtheriae*, is a 60-kDa polypeptide chain constituted by two fragments A and B linked to each other by a disulfide bridge. These fragments can be separated by site-specific cleavage of the protein and thiol reduction (Collier, 1975). After binding to yet unknown specific cell surface receptors (Middlebrook et al., 1978), the B fragment (M_r 37 240) mediates, via acidic endosomes (Sandvig & Olsnes, 1980; Marnell et al., 1984), the internalization of DT fragment A (M_r 21150) into the cytoplasm where it inhibits the protein synthesis and causes cell death by ADP ribosylation of elongation factor 2 (Pappenheimer, 1977). The importance of acidic pH for penetration of toxin into cells has been experimentally demonstrated

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; PE, phosphatidylethanolamine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine; egg PC, phosphatidylcholine from egg yolk; DPPA, DL-α-dipalmitoylphosphatidic acid; DPPC, DL-α-dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); SUV, small unilamellar vesicle(s); DT, diphtheria toxin; DTA, diphtheria toxin A fragment; DTB, diphtheria toxin B fragment; TX-100, Triton X-100; $C_{12}E_8$, octa(ethylene glycol) dodecyl ether; PTR, phase transition release; RET, resonance energy transfer; ANS, 1-anilino-8-naphthalenesulfonate; ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; DPX, *p*-xylenebis(pyridinium bromide).

0006-2960/89/0428-3406$01.50/0