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ADRIAMYCIN-LIPOSOME INTERACTIONS

A MAGNETIC RESONANCE STUDY OF THE DIFFERENTIAL EFFECTS OF CARDIOLIPIN ON DRUG-INDUCED FUSION AND PERMEABILITY

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Nuclear magnetic resonance and electron spin resonance spectroscopy were utilized to measure the effects of adriamycin on the fusion rates and permeability characteristics of dimyristoylphosphatidylcholine (DMPC) liposomes containing small amounts of other lipids. Liposomes of pure DMPC in the presence or absence of adriamycin at a molar ratio of 100:1 showed little or no fusion. Incorporation of up to 5% cardiolipin into DMPC liposomes increased the rate of fusion more than 200-fold. The addition of adriamycin to cardiolipin-containing liposomes further enhanced the rate of the fusion. In contrast, liposomes containing other phospholipids, including phosphatidylglycerol, sphingomyelin, and phosphatidylserine, were not as sensitive to the addition of adriamycin. The largest increase in the rate of fusion was observed when calcium ions were also present with adriamycin. Cardiolipin-containing liposomes again showed the greatest sensitivity. Adriamycin did not increase the permeability of DMPC liposomes to the paramagnetic ion, Pr^{3+}, either in the presence or absence of Ca^{2+}. However, in contrast, Pr^{3+} did gain access to the inside of cardiolipin-containing liposomes. Adriamycin decreased the rate of ascorbate permeation into the bilayer of dipalmitoylphosphatidylcholine (DPPC) liposomes and DPPC liposomes containing small amounts of phosphatidylglycerol or phosphatidylserine. Conversely, adriamycin stimulated the rate of ascorbate permeation of cardiolipin-containing DPPC liposomes. These results suggest that adriamycin can modulate membrane structure and function and act in a differential and specific way with membranes containing cardiolipin.

Introduction

Adriamycin, an important antineoplastic agent of the anthracycline class, interacts with the plasma membrane of cells [1–6] and with specific identifiable membrane components [7,8], particularly cardiolipin [9–13]. Examination of this phenomenon is of importance for two reasons. First, the plasma membrane is the initial barrier encountered by any drug and may be a critical determinant of therapeutic efficacy, regardless of the ultimate cellular target of the drug. Secondly, interaction of adriamycin with particular components of cellular membranes may be important for the expression of the cytotoxicity of the anthracyclines to neoplastic cells as well as for their toxicity to cardiac tissue [5,6,14–16].

Because of the structural complexity of the cell surface, we are utilizing phospholipid vesicles (i.e., liposomes) as a model to study the interaction of adriamycin with specific lipid components. We

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have previously reported a differential thermotropic effect of adriamycin on cardiolipin-containing liposomes as compared to other phospholipids [11,12]. The results suggest that the differential effect of adriamycin on cardiolipin-containing liposomes is due to an altered membrane organization when this lipid is present.

The present report utilizes magnetic resonance spectroscopy to examine the effect of adriamycin on (a) the fusion and (b) the permeability of liposomes with special attention to the role of the lipid composition. As before, we find that the presence of small amounts of cardiolipin in the bilayer confers a unique specificity to the membrane in its interaction with adriamycin.

Materials and Methods

Adriamycin (NSC 123127) was the gift of Dr. John D. Douros of the Division of Cancer Treatment, National Cancer Institute. Cardiolipin from bovine brain was obtained from Research Products, Miles Laboratories or Sigma Chemical Company; stearylamine from K and K Laboratories, and other lipids from Calbiochem or Sigma. All lipids were stored in a desiccator at −20°C. Their purity was ascertained by thin-layer chromatography on Silica gel using $CHCl_3/CH_3OH/H_2O$ (32.5:12:2, v/v). No impurities were detected by thin-layer chromatography in any of the samples used. No breakdown of cardiolipin was detected by thin-layer chromatography after longer periods of sonication than those described here.

Liposome solutions were prepared by standard sonication techniques [17–19] and contained 30–50 mg lipid/ml in ^2H_2O or phosphate-buffered saline, pH 7.4. Electron microscopic examination of sonicated preparations showed a fairly uniform size distribution with no observable multilamellar vesicles.

A Varian T60A 60 MHz continuous wave proton nuclear magnetic resonance (NMR) spectrometer was utilized to monitor fusion kinetics of the liposome preparations according to procedures developed in Prestegard's laboratory [20,21]. The resonance of the polar headgroup methyl protons is a well resolved single peak in the nuclear magnetic resonance spectrum. The fusion of small vesicles to form larger ones slows the molecular motion of the lipid. This in turn leads to resonance broadening, so that after extensive fusion has occurred, the motion of the molecules is so slow that no resonance is observed and the peak height decreases to zero. This technique has been shown to be primarily sensitive to vesicle fusion rather than to aggregation by Prestegard and his associates [20–22]; thus, the possibility of aggregation should not influence the results. During a kinetic experiment, the liposomes were maintained at 23°C (close to the transition temperature of the dimyristoylphosphatidylcholine vesicles) except for a warming to 37°C for 5 min prior to and during the 1-min sweep in the NMR spectrometer.

To determine the permeability of the liposome to ions, $PrCl_3$ was added to DMPC and cardiolipin-containing DMPC liposome preparations. The paramagnetic ion, Pr^{3+}, induces a downfield shift in the resonance position of the choline methyl protons in the outer leaflet of the liposomal bilayer [23]. If the permeability to cations is increased (for example by drug treatment) and Pr^{3+} enters the liposomes, the inner choline methyl protons are also shifted and the splitting collapses. The time-course of the degree of resonance splitting reflects changes in the permeability of the lipid bilayer to Pr^{3+}.

A second measure of membrane permeability employed the rate of reaction of ascorbate with the spin label 5-doxylstearic acid incorporated into liposomes [24,25]. Liposomes were prepared as described for the NMR experiments, except that 1% 5-doxylstearic acid was included in the lipid mixture. The ESR spectrum of liposomes containing a spin label is typical of anisotropic probe motion in membrane bilayers. To follow the rate of reduction, the spectrometer magnetic field was swept to the center line of the spectrum and held at that position. The intensity of the paramagnetic resonance signal then was recorded as a function of time. No resonance of an ascorbate free radical is observed under these conditions nor does ascorbate reduce adriamycin as determined by visible spectroscopy.

Results

The effect of increasing levels of cardiolipin on the rate of fusion of DMPC liposomes is shown in

TABLE I

EFFECT OF INCREASING LEVELS OF CARDIOLIPIN ON THE FUSION OF DMPC LIPOSOMES AT 23°C

DMPC liposomes were prepared as described in Methods and contained increasing amounts of cardiolipin as indicated. The values for the rate of fusion were determined from a least-squares fit of the data to a first order process.

Liposome composition	Rate contant $k(h^{-1})(\times 10^3)$
DMPC	2.49
DMPC+0.05 mol% cardiolipin	2.67
DMPC+0.13 mol% cardiolipin	4.75
DMPC+0.25 mol% cardiolipin	7.61
DMPC+1.0 mol% cardiolipin	14.8
DMPC+5.0 mol% cardiolipin	>530

Table I. The rate of fusion increased from a value similar to that obtained with pure DMPC liposomes when a concentration of 0.05% cardiolipin was employed to a rate 200-times faster when 5.0% cardiolipin was present in the liposomes.

A comparison of the effects of adriamycin and Ca^{2+} on DMPC and cardiolipin-containing DMPC liposomes is shown in typical plots in Figs. 1a and 1b, respectively. Adriamycin had little or no effect on the rate of fusion of pure DMPC liposomes. In contrast, a pronounced increase in the rate of fusion was observed in DMPC liposomes containing cardiolipin when exposed to 10^{-4} M adriamycin. The molar ratio of lipid to adriamycin was approx. 100:1. The addition of 1 mM Ca^{2+} had little or no effect on the cardiolipin-containing DMPC liposomes and only affected pure DMPC liposomes after lengthy exposure. Consistently, with both DMPC and cardiolipin-containing DMPC liposomes, there appeared to be a synergistic effect on the fusion rate in the simultaneous presence of both adriamycin and Ca^{2+}, with the effects being most pronounced in cardiolipin-containing DMPC vesicles.

The effects of adriamycin and Ca^{2+} on DMPC liposomes containing other lipids were also evaluated to determine if the observed phenomenon was unique to cardiolipin. Phosphatidylglycerol is a phospholipid that is structurally one-

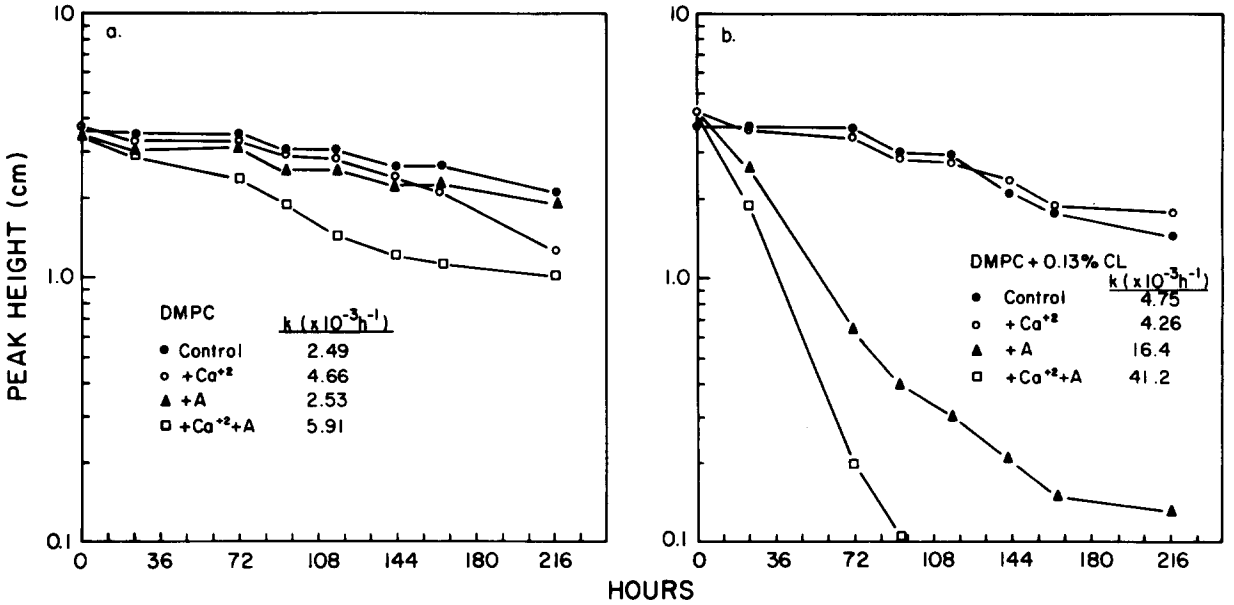

Fig. 1. The effects of Ca^{2+}, adriamycin (A), and Ca^{2+} plus adriamycin on the fusion of DMPC liposomes (a) and DMPC liposomes containing 0.13% cardiolipin (b). The ^1H peak height of the choline methyl group is plotted against time. The concentrations of Ca^{2+} and adriamycin were 1 mM and 0.1 mM, respectively. The rates of fusion were determined from a least-squares fit of the data to an exponential decay as described in Materials and Methods.

half of cardiolipin, while still maintaining a net negative charge; phosphatidylserine also represents a negatively charged phospholipid but is not as closely related in structure to cardiolipin; and sphingomyelin is a neutral phospholipid. The addition of Ca^{2+} to phosphatidylglycerol-containing

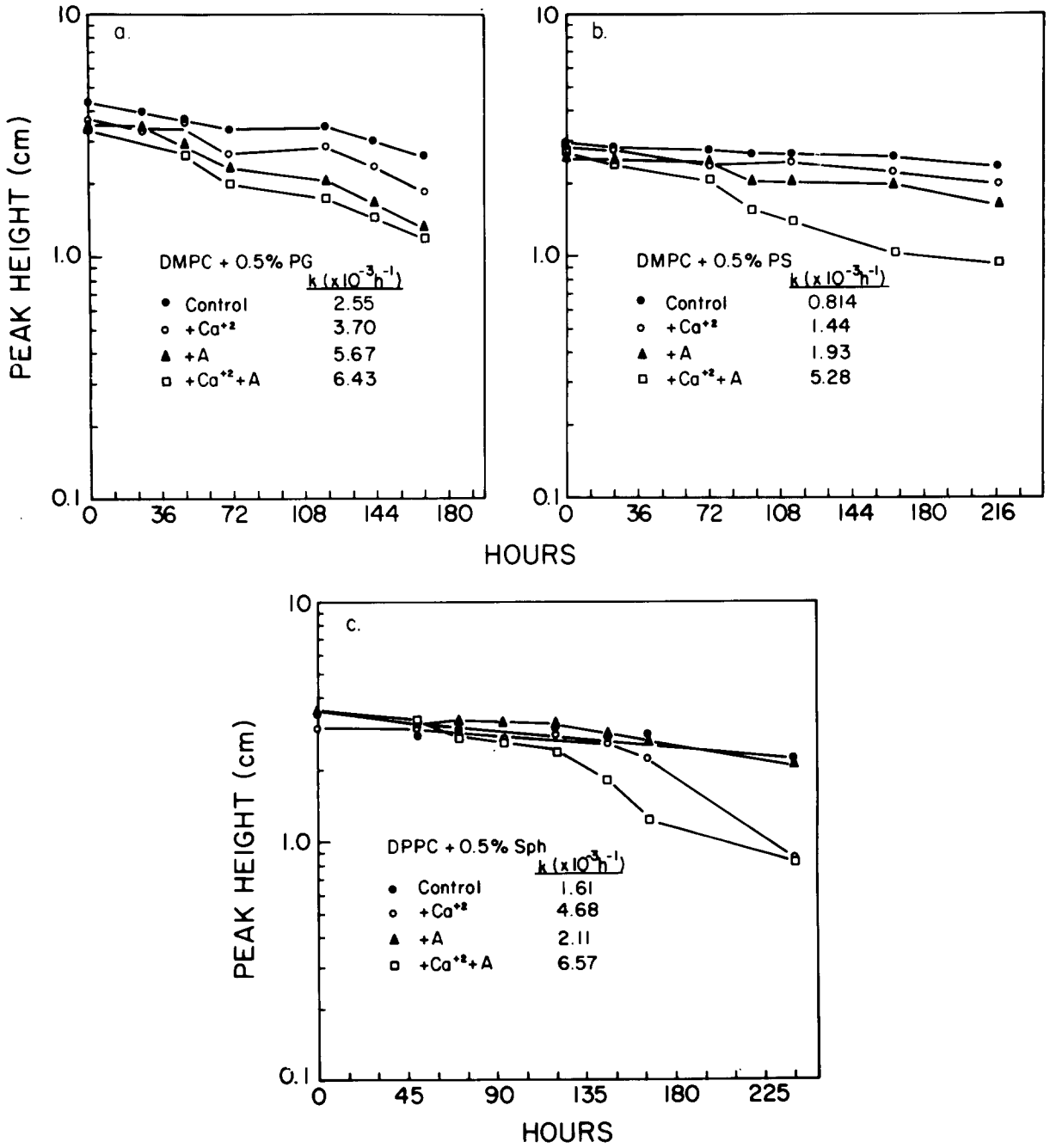

Fig. 2. The effects of Ca^{2+}, adriamycin (A), and Ca^{2+} plus adriamycin on the fusion of DMPC liposomes containing 0.5% phosphatidylglycerol (a), 0.5% phosphatidylserine (b), or 0.5% sphingomyelin (c). The concentrations of adriamycin and Ca^{2+} and the method used for the calculation of the rate constants are given in Fig. 1.

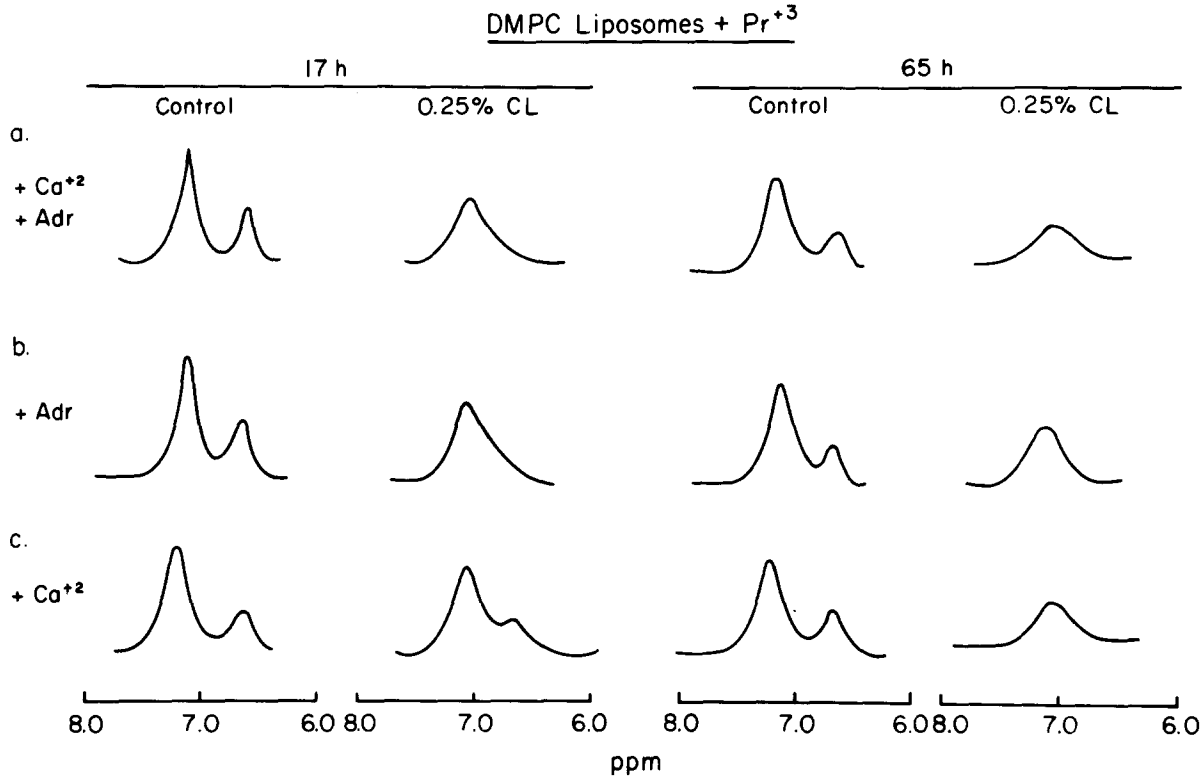

Fig. 3. NMR spectra of DMPC liposomes and DMPC liposomes containing 0.25% cardiolipin (CL) in the presence of Pr^{3+}. Spectra show the effects of (a) 1 mM Ca^{2+} and 0.1 mM adriamycin (Adr), (b) 0.1 mM adriamycin, and (c) 1 mM Ca^{2+} after 17 h and 65 h of exposure. The location of the resonance signals is given in parts per million from tetramethyl silane. Spectra of control liposomes and 0.25% cardiolipin containing liposomes with no addition of Ca^{2+} or adriamycin resembled the spectra of added Ca^{2+} in all cases.

liposomes enhanced the rate of fusion only slightly (Fig. 2a). The addition of adriamycin caused about a 2-fold increase in the rate of fusion and, as before, the largest effect was observed in the presence of both adriamycin and Ca^{2+}. The results, however, do not show nearly the same extent of enhancement of fusion as exhibited by cardiolipin-containing liposomes in the presence of adriamycin or adriamycin and Ca^{2+}. Phosphatidylserine-containing liposomes showed a small increase in the rate of fusion with the addition of Ca^{2+}, a slightly larger increase in the presence of adriamycin, and the largest effect when both adriamycin and Ca^{2+} were present simultaneously (Fig. 2b). Again, the results obtained with phosphatidylserine-containing liposomes did not approach the enhanced rates of fusion observed with cardiolipin-containing liposomes. The results obtained with sphingomyelin were nearly identical to those observed with pure DMPC liposomes (Fig. 2c). In all cases, the addition of both adriamycin and Ca^{2+} produced the largest increase in the rates of fusion, with cardiolipin-containing liposomes consistently experiencing the most dramatic enhancement of the rate of fusion in the presence of the anthracycline.

Since the negatively charged phospholipids, phosphatidylglycerol and phosphatidylserine, did not enhance the rate of fusion in a manner comparable to that of cardiolipin-containing liposomes, the effect of cardiolipin appears to be the result of its molecular structure rather than its negative charge.

Permeability of liposomes to Pr^{3+}

To determine the effect of adriamycin on cation permeability into liposomes, we have utilized the paramagnetic ion, Pr^{3+}, which is relatively imper-

TABLE II

EFFECT OF ADRIAMYCIN AND CARDIOLIPIN ON THE PERMEABILITY OF DIPALMITOYLPHOSPHATIDYLCHOLINE (DPPC) LIPOSOMES

A concentration of 23 mM ascorbate in 50 μl of liposome preparation resulted in half-times of 1 to 3 min depending upon the sample. As expected, the absolute values of the rate of reduction of 5-doxylsteric acid by ascorbate was faster at 50°C for both DPPC and cardiolipin-containing DPPC liposomes. The ratios, however, of the absolute rates in the presence and absence of adriamycin (0.1 mM) were similar for the two temperatures. Adr, adriamycin. υ_{red}, rate of reduction.

Condition	$\frac{\upsilon_{red}(+Adr)}{\upsilon_{red}(-Adr)}$
DPPC, 25°C ($<T_m$)	0.775
DPPC, 50°C ($>T_m$)	0.669
DPPC + 1 mol% cardiolipin, 25°C	2.17
DPPC + 1 mol% cardiolipin, 50°C	2.15
DPPC + 2 mol% phosphatidylglycerol, 25°C	0.745
DPPC + 2 mol% phosphatidylserine, 25°C	0.702

meable to liposomes. Exposure of liposomes to Pr^{3+} produces a splitting of the choline methyl protons into inner bilayer (unshifted by the reagent) and outer bilayer (shifted downfield by Pr^{3+}) signals. If the liposomal membrane becomes more permeable to Pr^{3+}, the ion reaches the interior of the liposomes, the inner bilayer signal is shifted downfield, and the split signal collapses. Fig. 3 shows that this indeed occurs in response to cardiolipin. At 17 h, two peaks are very clearly present in pure DMPC liposomes in the presence of adriamycin and/or Ca^{2+}. In contrast, at 17 h in the cardiolipin-containing liposomes, the two peaks have collapsed into one peak in the presence of adriamycin or adriamycin and Ca^{2+}. In the presence of Ca^{2+} alone, two peaks are still present though not as sharply distinguishable as with pure DMPC liposomes. After 65 h, very little change occurred in the pure DMPC liposomes. The presence of Ca^{2+} and/or adriamycin did not cause an increase in the permeability of the pure DMPC liposomes. At 65 h, the cardiolipin-containing liposomes showed only one peak under all conditions examined.

Permeability of liposomes to ascorbate

To further examine the permeability of cardiolipin-containing liposomes and to ascertain the possible effects of adriamycin, liposome preparations containing the fatty acid spin label 5-doxylstearic acid were employed. The paramagnetic center of the probe is reduced by ascorbate at a rate dependent upon its ability to penetrate the bilayer. Since the kinetics are first order, the rate constant for the process is a relative measure of ascorbate permeability [24,25]. The results of the ascorbate permeability studies are summarized in Table II. In each case the data are presented as the ratio of the rate constant in the presence and absence of adriamycin. Ratios less than one indicate that the drug slowed the rate of ascorbate penetration. Conversely, ratios greater than one indicate that the drug increased the rate of ascorbate penetration. With pure dipalmitoylphosphatidylcholine (DPPC) liposomes (T_m approx. 41°C), the drug caused a decrease in the rate of ascorbate permeability whether the lipid bilayer was in the gel (below the T_m) or liquid crystalline (above the T_m) phase. Adriamycin was present at a concentration of one to two molecules per liposome. Thus, the drug had a pronounced effect on membrane structure, since it caused a significant decrease in the ability of ascorbate to penetrate to the 5 position of the fatty acid chain of 5-doxylsteric acid. When small amounts of either phosphatidylglycerol or phosphatidylserine were incorporated into the phosphatidylcholine matrix, the drug again caused the rate of reduction to be slower than that occurring when no drug was present. The introduction of 1% cardiolipin, however, had exactly the opposite effect. Adriamycin stimulated the rate of ascorbate reduction of the spin probe. Thus, the anthracycline modulated membrane permeability in each case examined, but the effect was uniquely different when cardiolipin was present.

Discussion

We have demonstrated that the antineoplastic agent adriamycin is capable of modulating three important membrane physical properties: fluidity [6,11], fusion, and permeability. Since these and several other biological membrane properties are affected by this drug [1–8], direct membrane actions may be involved in the mechanism of cyto-

toxicity of the anthracycline. We have addressed the possibility that lipid composition, especially the presence or absence of cardiolipin, may confer some specificity on the interaction of adriamycin with membranes. This possibility is supported by published work from this and other laboratories [9–13,26].

To provide a relatively simple system for investigating the interaction of adriamycin with membrane lipids, we have employed the liposome as a model for a cellular membrane whose composition can be varied at will. DMPC or DPPC were used to provide a well-defined and homogeneous host matrix. Thus the effects of adriamycin and Ca^{2+} and the insertion of precise amounts of other phospholipids on liposome behavior were observed in a well-known environment. In the present studies of liposome fusion, it is clear that adriamycin-induced effects on this process are dependent upon the phospholipid composition. Pure phosphatidylcholine liposomes, for example, do not undergo significant fusion over a time course of several days and cannot be induced to do so either by the known fusogen, Ca^{2+}, or by adriamycin. The introduction of small molar amounts of cardiolipin, however, renders these liposomes susceptible to fusion. This observation is not surprising because acidic phospholipids are known to be more fusogenic than neutral ones [18]. However, the ability of adriamycin to greatly stimulate the fusion rate of cardiolipin-containing liposomes is unique, both because Ca^{2+} itself does not have this ability (Fig. 1b) and because the effect is limited only to cardiolipin, and not to acidic phospholipids as a class.

A synergistic effect of Ca^{2+} and Mg^{2+} on the fusion of phosphatidylserine vesicles was reported by Portis et al. [27]. They proposed that the observed synergism between the two ions is related to the reduction of the surface charge density by Mg^{2+} which allows for the formation of the phosphatidylserine/Ca complex at low concentrations of Ca^{2+} because of the enhanced possibility for close contact between vesicles. The Mg^{2+} may lower the threshold concentration of Ca^{2+} required for fusion. The apparent synergism observed between adriamycin and Ca^{2+} in the induction of fusion in the present study extended to all of the liposomes examined although the greatest effect occurred with cardiolipin-containing liposomes. Smaller degrees of synergism were observed in pure DMPC liposomes and DMPC liposomes containing phosphatidylglycerol, phosphatidylserine, and sphingomyelin. Since both neutral and negatively charged phospholipids exhibited some synergism, the effect does not appear to be related to net charge, but rather to configuration. We have previously provided evidence that liposomes containing small amounts of cardiolipin present a different surface to adriamycin than liposomes lacking this component [11,12]. Goormaghtigh et al. [26] have recently provided evidence that the ability of cardiolipin to stimulate non-bilayer structures in membranes is antagonized by adriamycin.

One of the central functions of a biological membrane is the ability to serve as a permeability barrier. Liposomes also display this property and therefore can be employed as a model for factors affecting cell membrane permeability. The NMR results using Pr^{3+} show that the paramagnetic ion is not permeable in pure DMPC liposomes, either in the presence or absence of adriamycin. The incorporation of cardiolipin into the phosphatidylcholine matrix, however, allows adriamycin to stimulate the passive diffusion of Pr^{3+} across the bilayer on a time-scale which is faster than membrane fusion. Thus, fusion itself should not lead to large scale internalization of Pr^{3+} which is initially confined to the liposome exterior. Consequently, we conclude that the ion is actually crossing the membrane barrier rather than gaining access to the interior of the liposomes by a fusion mechanism which is leaky. Other investigators have reported that adriamycin alters cation distributions (both mono- and divalent) in intact cells [28–31], although the mechanisms involved have not been identified. The present work suggests that cation permeability may be influenced by adriamycin solely through lipid interactions and that protein carriers are not necessarily involved.

ESR studies were undertaken to determine if adriamycin was capable of altering anion (ascorbate) permeability. The experiments reveal that ascorbate penetration into the liposomal bilayer is very sensitive to adriamycin under all conditions studied. Pure DPPC liposomes and DPPC liposomes containing phosphatidylglycerol or phos-

phatidylserine exhibit a decrease in their permeability to ascorbate when adriamycin is present. The inclusion of cardiolipin in the bilayer, however, causes an increased permeability of ascorbate in the presence of adriamycin.

Butler [25] used the free radical reduction technique to investigate the effects of local anaesthetics on ascorbate permeability of liposomes. His findings showed that tetracaine caused an increase in the permeability of egg lecithin liposomes under conditions in which there were about 108 drug molecules per vesicle. A much lower concentration of drug was employed in the experiments with adriamycin described in this report, and the effects on permeability are evident when only about 2 molecules of adriamycin are bound to each liposome. This is surprising because it suggests that the drug has a long range effect on membrane structure. Thus, when only 2 molecules of adriamycin are bound to a liposome, all of the nitroxide sites (approx. 25 per liposome) sense that the drug is present. Since the reduction kinetics can be fit to a single experimental decay and since none of the ESR signal remains after the reduction process, we conclude that the membrane structural change provoked by adriamycin is felt at all locations on the liposome, regardless of their proximity to the bound drug. Thus, the effect of the binding of adriamycin is propagated throughout the bilayer. This is in contrast to the case of proteins embedded in membranes, where spin-labeling results suggest that the presence of the protein molecule is only experienced at the immediately adjacent phospholipid (boundary lipid) and not further into the membrane [32].

These results and previous observations by us and others show that membrane properties can be modulated by the antineoplastic agent adriamycin. This is not meant to be taken as proof for a role of the membrane in the mechanism of cytotoxicity produced by the drug, but rather to suggest that it is important to consider functional targets other than DNA for the action of adriamycin. Furthermore, these results add to the growing body of evidence [9–13,26] that adriamycin acts in a differential and specific way with membranes that contain cardiolipin. Such interaction may be involved either in the mechanism of action or the specificity of action of this and related agents. In either case, there exists the potential of exploiting this phenomenon to enhance the pharmacologic action of this important drug.

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