Biochemical Pharmacology, Vol. 34, No. 24, pp. 4291–4298, 1985.
Printed in Great Britain.

0006–2952/85 $3.00 + 0.00

INTERACTION OF Ca^{2+} WITH CARDIOLIPIN-CONTAINING LIPOSOMES AND ITS INHIBITION BY ADRIAMYCIN

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(*Received* 23 *October* 1984; *accepted* 20 *May* 1985)

Abstract—The interaction of cardiolipin-containing, unilamellar liposomes with Ca^{2+} was assessed by flow dialysis in the presence of 2–100 μM $^{45}Ca^{2+}$, using vesicles formed from phosphatidylcholine (PC) and from PC and cardiolipin in mole ratios from 16:1 to 1:1. Control (PC only) vesicles bound no detectable Ca^{2+}. In contrast, Ca^{2+} binding to cardiolipin-containing vesicles was substantial and dependent on vesicle concentration. Scatchard plots for the binding were concave upward. Resolution of the data, assuming the presence of two independent classes of binding sites, indicated a high-affinity site with apparent $K_D = 5.57 \pm 0.48$ μM (S.D.) and a second site with K_D in the millimolar range. Interaction of cardiolipin-containing liposomes with Ca^{2+} was insensitive to monovalent cations (Na^+, K^+, Rb^+), but was inhibited by ruthenium red $\gg La^{3+} > Mn^{2+} > Mg^{2+}$. Progressive increases in the PC:cardiolipin ratio markedly increased the apparent K_D for Ca^{2+} at the high-affinity site. Stoichiometry of Ca^{2+} binding at the site passed through a maximum at a PC:cardiolipin ratio of 4:1. The potent antineoplastic agent adriamycin also inhibited the interaction of Ca^{2+} with cardiolipin-containing liposomes in a dose-dependent manner; effects were detected at 10 μM antibiotic. Unlike PC, adriamycin altered the stoichiometry of the high-affinity interaction but not the apparent K_D. Adriamycin effects increased with pH in the range of the pK_A of its amino group. These results suggest that inhibition by adriamycin may result from a mechanism other than simple competition for the charged head group of cardiolipin.

Adriamycin (doxorubicin) is a potent antineoplastic agent [1, 2], but its clinical use is limited by cumulative cardiotoxicity [3, 4]. The oncolytic activity of the drug is attributed to intercalation of the anthracycline ring into the DNA double helix [1, 5], possibly followed by localized generation of free radicals [6]. The biochemical basis of its cardiotoxic side effects is much less clear [7]. One potential intracellular target of adriamycin is cardiolipin, an acidic phospholipid with which the drug interacts strongly ($K_A = 1.6 \times 10^6$ M^{-1}; [8, 9]).

Cardiolipin (diphosphatidylglycerol) is an unusual phospholipid containing four fatty acids and a head group with two negatively charged phosphate groups [10]. In eukaryotic cells, cardiolipin is restricted primarily to the mitochondrial membranes [11], where it is a major component. Cardiolipin accounts for 17 and 20% of the phospholipid content (by weight) of mitochondria from bovine liver and heart respectively [12]. All but trace amounts of mitochondrial cardiolipin is found in the inner membrane [13–15], and more refined analyses of the heart preparation localize 75% of the inner membrane cardiolipin to the bilayer leaflet facing the matrix [15].

Cardiolipin may play several roles in the mitochondrial membrane. A requirement for cardiolipin in electron transport through cytochrome *c* oxidase [16–18] and electron transfer complexes I and II [19] has been established. Cardiolipin is also required for optimal function of the reconstituted mitochondrial phosphate translocator [20, 21]. Sensitivity of both electron transport [22] and the phosphate translocator [23] to adriamycin has been reported.

Several observations support the proposition that cardiolipin may participate in movement of Ca^{2+} across the inner mitochondrial membrane [24, 25]. (1) Cardiolipin facilitates the sequestration of Ca^{2+} in the organic phase of a two-phase extraction system [24–26]. (2) Cardiolipin has been reported to mediate the movement of Ca^{2+} through a bulk stirred organic phase from one aqueous phase to another [27]. (3) Addition of cardiolipin to platelet suspensions promotes an influx of Ca^{2+} and a related release of serotonin [28]. (4) Ca^{2+} induces cardiolipin to adopt inverted, non-bilayer structures potentially suitable for Ca^{2+} sequestration [29, 30]. However, attempts to measure cardiolipin-dependent Ca^{2+} uptake into multilamellar liposomes containing up to 5 mole percent cardiolipin have met with little success [31, 32].

We have focused recently on the possible role of cardiolipin in Ca^{2+} translocation, suggesting that, in order to function in Ca^{2+} transport across an intracellular membrane, cardiolipin must be able to interact with Ca^{2+} at physiological, cytosolic concentrations (0.1 to 10 μM, see Ref. 33) [34]. (This restriction would be somewhat relaxed if cardiolipin were to mediate Ca^{2+} efflux from mitochondria.) Because all earlier studies of Ca^{2+}-cardiolipin interaction had used Ca^{2+} concentrations in the millimolar range, we re-examined the interaction in a model two-phase organic extraction system using micromolar Ca^{2+} and phospholipid concentrations. Those experiments allowed us to identify Ca^{2+}-cardiolipin interactions of high affinity (apparent K_D for Ca^{2+} = 1–3 μM, depending on organic solvent), characterized by cation selectivity. Phosphatidylcholine

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(PC) was found to inhibit cardiolipin-mediated extraction of Ca^{2+} into the organic phase [34]. Extraction was also inhibited by adriamycin [35].

We have now extended our studies to examine the interaction of Ca^{2+} with cardiolipin-containing unilamellar vesicles prepared by a variant [36] of the detergent-dialysis method [37]. The properties of Ca^{2+}-cardiolipin interaction observed in the two-phase organic extraction system—high affinity, cation selectivity, and sensitivity to PC—were preserved when cardiolipin was incorporated into a phospholipid bilayer in contact with an aqueous solution at physiological pH. Furthermore, the interaction was inhibited in a dose-dependent fashion by low concentrations ($<100\ \mu M$) of adriamycin.

MATERIALS AND METHODS

Formation of phospholipid vesicles. Unilamellar phospholipid vesicles were formed from PC and cardiolipin according to Mimms *et al.* [36]. Solvents were removed from phospholipids (10 μmoles) by evaporation under nitrogen. The lipids were washed twice with petroleum ether and evacuated for at least 30 min to remove all traces of solvent. Detergent (0.5 ml of 0.3 M octylglucoside in 10 mM Hepes*-KOH, pH 7.4; final phospholipid concentration = 20 mM, detergent/lipid ratio = 15:1) was added, and the mixture was applied to a Sephadex G-50 column (1.5 × 20 cm). The column was eluted at a flow rate of 1.2 ml/min with 10 mM Hepes-KOH, pH 7.4, that had been de-gassed. (Unless otherwise noted, all manipulations were carried out at room temperature in this buffer.) Vesicles always emerged in the column void volume in a sharp (<3 ml) peak. Their position was identified by clearly visible turbidity for the higher PC:cardiolipin ratios and by u.v. light scattering for vesicles containing higher mole fractions of cardiolipin. Phospholipid analyses [38] of the column fractions confirmed the position of vesicle emergence. More than 80% of the lipids applied to the column were recovered in the pooled vesicle fractions for PC:cardiolipin ratios > 2:1 (mole/mole). For higher cardiolipin content, vesicle yield was somewhat reduced.

Phospholipid vesicles were formed from mixtures of PC and cardiolipin in several PC:cardiolipin mole ratios: 1:1, 2:1, 4:1, 8:1, 12:1, 16:1, and 1:0. Lipids were stored at −15°; vesicles were stored at 5° and used within 1 week, during which time no changes in Ca^{2+} binding were observed (see also Ref. 37). Vesicle composition was compared to the composition of the phospholipid mixtures from which the vesicles were formed by thin-layer chromatography on silica gel G plates. Plates were developed in chloroform–methanol–water (65:25:4), and spots were visualized by spraying with 50% H_2SO_4 and charring. No evidence for selective phospholipid incorporation into the vesicles was obtained; in all cases, TLC profiles for vesicles matched (qualitatively) those for the corresponding PC/cardiolipin mixtures. No octylglucoside could be detected in any of the vesicle preparations. Measurements using [^{14}C]octylglucoside [36] suggest that less than 0.01% of the detergent is retained by the vesicles.

Measurement of calcium binding. Binding of $^{45}Ca^{2+}$ to the vesicles was measured by means of flow dialysis [39] using a custom-constructed Teflon apparatus with an inner diameter of 1.0 cm. The upper and lower portions of the chamber were separated by a 3500 molecular weight cut-off dialysis membrane. The contents of both compartments were stirred. The volume of the upper phase was 2.0 ml. In initial experiments, buffer was pumped through the 0.25 ml lower portion of the chamber at a rate of 0.44 ml/min, and 1-min fractions of the effluent were collected directly into scintillation vials. In later experiments examining adriamycin effects, flow rate was increased to 1.10 ml/min and 30-sec fractions were collected. Aliquots (0.4 ml) were then transferred to vials. The relative $^{45}Ca^{2+}$ content of each vial was determined by standard liquid scintillation counting. Similar results were obtained with both protocols. For binding interactions of sufficient strength, the flow dialysis method permits estimation of number and affinity(s) of binding sites based on data obtained with a single sample over the course of an hour.

Data from two typical flow dialysis determinations of $^{45}Ca^{2+}$ binding to PC/cardiolipin vesicles are plotted in Fig. 1. Results were analyzed as previously described [39], correcting for a Ca^{2+} concentration of 1.16 μM in the Hepes buffer, determined by atomic absorption spectrophotometry. The experiments reported here meet several key criteria. (1) The permeability to Ca^{2+} of the dialysis membrane separating the two portions of the chamber does not change during the course of the experiment. In control experiments carried out in the absence of vesicles, the ratio of the steady-state concentration of $^{45}Ca^{2+}$ in the effluent stream to $^{45}Ca^{2+}$ concentration in the upper phase was invariant for total Ca^{2+} concentrations from 1 to 100 μM and for all adriamycin concentrations employed (data not shown). (2) Loss of Ca^{2+} from the upper chamber during the course of an experiment is minimal (<15% of the label initially present). (3) The Ca^{2+} concentration in the buffer stream flowing through the lower chamber reaches a steady state after each addition of non-radioactive Ca^{2+} (Fig. 1).

Data analysis requires estimation of cpm_{MAX}, the steady-state concentration of $^{45}Ca^{2+}$ in the effluent when no $^{45}Ca^{2+}$ is bound to the vesicles. In theory, cpm_{MAX} would be determined by adding a sufficiently large excess of cold Ca^{2+} to displace all bound $^{45}Ca^{2+}$. However, permeability of the dialysis membrane appears to decrease at elevated Ca^{2+} concentrations. We have therefore used addition of 10 μM ruthenium red to define cpm_{MAX}.

Materials. $^{45}Ca^{2+}$ was purchased from New England Nuclear. Bovine heart cardiolipin (sodium salt), egg yolk phosphatidylcholine (Type XI-E), octylglucoside (*n*-octyl-β-D-glucopyranoside), ruthenium red, lanthanum chloride, and Hepes were purchased from Sigma; Sephadex G-50 (fine) was from Pharmacia, and silica gel G TLC plates from Fisher Scientific. Adriamycin-HCl was provided by Dr. N. Bachur, University of Maryland Cancer Research

* Abbreviations: Hepes, *N*-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid; PC, phosphatidylcholine; and PE, phosphatidylethanolamine.

Center. All other chemicals were of reagent grade. Ruthenium red concentration was determined spectrophotometrically [40]. The sample was essentially free of contamination by ruthenium brown or ruthenium violet. Spectrapor 3 dialysis membranes (Spectrum Medical Industries, Los Angeles, CA) were stirred for several hours in the presence of 30 mM $NaHCO_3$ and 1 mM ethyleneglycolbis-(amino-ethylether)tetra-acetate (EGTA), and then washed extensively with distilled water. Membranes were stored at 4° in 50% ethanol until use.

RESULTS

Vesicles formed from PC and cardiolipin in a 4:1 ratio bound significant amounts of Ca^{2+} under these experimental conditions (Fig. 1). In contrast, results obtained with vesicles formed from PC only were indistinguishable from controls in which the upper phase contained buffer only (data not shown). That is, no binding of Ca^{2+} to PC vesicles or to any residual detergent they may contain could be detected.

A Scatchard plot for the binding of Ca^{2+} to PC/cardiolipin (4:1) vesicles (Fig. 1, inset) shows clear upward curvature. Such a plot will always be produced by interaction of a ligand with multiple classes of binding sites; it can also result from negative cooperativity in binding [41]. We have assumed the presence of two classes of binding sites, resolving the

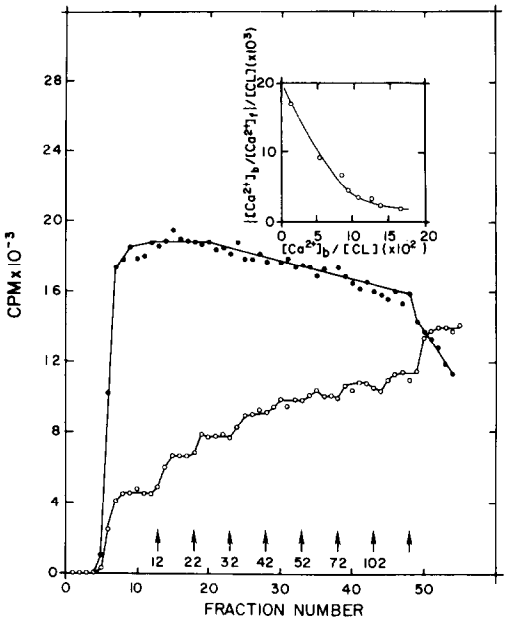

Fig. 1. Flow dialysis determination of Ca^{2+} binding to PC/cardiolipin vesicles. The 2.0 ml upper phase contained vesicles equivalent to 1.18 μmoles phospholipid, formed from PC/cardiolipin (4:1, mole/mole; open symbols) or PC only (closed symbols). $^{45}Ca^{2+}$ (2 μl containing 2 nmoles Ca^{2+}) was added to the upper chamber as collection of fraction 5 began. At the points indicated by the arrows, sequential additions of non-radioactive 10 mM $CaCl_2$ were made to produce the indicated total (micromolar) Ca^{2+} concentrations. At fraction 48, 4 μl of a 5 mM ruthenium red stock solution was added. The cpm in 1-min fractions of the buffer flow through the lower portion of the chamber are plotted on the ordinate. Inset: a Scatchard plot for Ca^{2+} binding to PC/cardiolipin (4:1) vesicles. $[Ca^{2+}]_b$ and $[Ca^{2+}]_f$ were calculated according to Colowick and Womack [39]. The value of cpm_{max} was defined by addition of ruthenium red. After each addition, the steady-state value of cpm/cpm_{max} was taken as an indication of the proportion of the total Ca^{2+} in the upper chamber that was free. The line is an algebraic fit assuming two classes of binding site [42]. (The decrease in $^{45}Ca^{2+}$ in the effluent stream observed upon ruthenium red addition to PC vesicles reflects a decrease in dialysis membrane permeability, induced by free ruthenium red, that did not occur when cardiolipin-containing vesicles were used.)

Table 1. Effect of vesicle concentration on Ca^{2+} binding by PC/cardiolipin vesicles in the presence of 2 μM Ca^{2+}

Vesicle preparation	Volume of vesicles added to assay (ml)	$[Ca^{2+}]_b$ (μM)
PC/cardiolipin (4:1)	0.1	0.65
	0.2	1.04
	0.4	1.56
	0.6	1.84
PC/cardiolipin (8:1)	0.4	0.84
	0.5	0.99
	0.8	1.32

Data were obtained from plots of the type shown in Fig. 1. $[Ca^{2+}]_b$ was calculated from the rate of $^{45}Ca^{2+}$ appearance in the buffer flow stream at vials 11–13, i.e. prior to the addition of cold Ca^{2+}. Total phospholipid concentration in the PC/cardiolipin vesicle preparations was 3.0 mM.

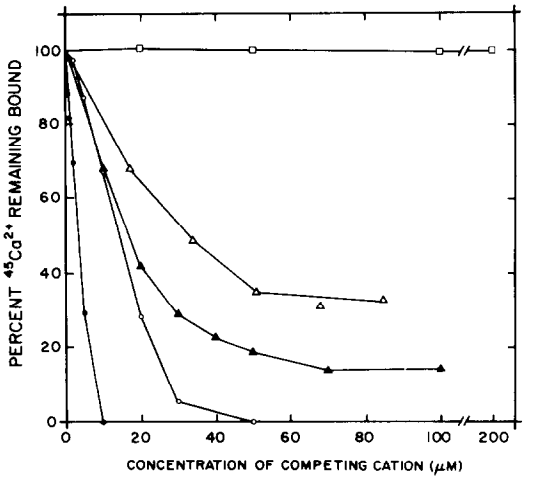

Fig. 2. Displacement of $^{45}Ca^{2+}$ bound to PC/cardiolipin (4:1) vesicles by various cations. Determinations were made using a flow dialysis protocol similar to that in Fig. 1. The upper chamber contained vesicles equivalent to 118 μM cardiolipin (114 μM in the experiment using La^{3+}). At fraction 5, 1 μM $^{45}Ca^{2+}$ was added to the upper phase. At fraction 13 and at subsequent 5-vial intervals, additions of the competing cation were made. The measurements were terminated by addition of 10 μM ruthenium red to yield cpm_{max}. The cpm_{max} value obtained was the same for all runs. After each addition, the steady-state cpm value was used to calculate the fraction of the $^{45}Ca^{2+}$ still bound as $(1\text{-}cpm/cpm_{max})$. Values are plotted as a percentage of the Ca^{2+} bound in the absence of competing cation. Data shown are for ruthenium red (●), $LaCl_3$ (○), $MnCl_2$ (▲), $MgCl_2$ (△), and NaCl(□).

Table 2. Effect of vesicle composition on binding constants for the interaction of Ca^{2+} with PC/cardiolipin vesicles

PC/Cardiolipin ratio	High-affinity site		Low-affinity site	
	K_D (μM)	n/CL	K_D (mM)	n/CL
1:1	2.68	0.030	0.56	1.48
2:1	3.53	0.075	7.02	23.3
4:1	5.00	0.092	2.71	2.7
8:1	6.34	0.081	0.57	0.43
12:1	7.66	0.051	1.09	0.57
16:1	10.73	0.042	0.85	0.50

Binding constants were derived as recommended by Rosenthal [42] from the data shown in Fig. 3. For each vesicle preparation, the two straight lines specified by the binding constants were summed vectorially to yield the curves in Fig. 3. The data for PC/cardiolipin 1:1 vesicles are not included in Fig. 3; those data generated a curve that crossed several of the others, rendering the graph undecipherable.

data according to Rosenthal [42]. This approach yielded K_D values of 5.0 μM and 2.7 mM for the high- and low-affinity sites, respectively, and generated the curve shown in the inset. Flow dialysis measurements on four separate PC/cardiolipin (4:1) vesicle preparations produced a mean K_D of 5.57 ± 0.48 μM (S.D.) for the high-affinity site and indicated the presence of 0.101 ± 0.007 Ca^{2+} binding sites per cardiolipin. Estimates of the binding constants for the assumed low-affinity site were far more variable: mean apparent K_D = 26.0 mM (range: 2.7 to 70.2 mM), mean number of sites per cardiolipin = 26.0 (range: 2.7 to 70.2). Regardless of the interpretation applied to the data, however, it is clear that, even at 2 μM total Ca^{2+}, the amount of Ca^{2+} bound by the vesicles was substantial and depended on the concentration of vesicles in the upper chamber (Table 1).

Selectivity of Ca^{2+}-cardiolipin interaction was assessed by comparing the abilities of various cations to displace $^{45}Ca^{2+}$ bound to PC/cardiolipin (4:1) vesicles. For four separate vesicle preparations, 50% displacement of $^{45}Ca^{2+}$ required 28.5 ± 5.2 (S.D.) μM Ca^{2+}. As shown in Fig. 2, the effectiveness of other cations decreased in the order ruthenium red > La^{3+} > Mn^{2+} > Ca^{2+} > Mg^{2+} ≫ Na^+. K^+ and Rb^+ were also without effect under these conditions (data not shown).

In a two-phase organic extraction system, the interaction of cardiolipin with Ca^{2+} is inhibited by PC [34]. The effects of increasing PC content on the ability of PC/cardiolipin vesicles to bind Ca^{2+} are exemplified by the normalized Scatchard plots of Fig. 3. Vesicle concentration in the flow dialysis assay was adjusted so that all preparations bound equivalent amounts of the input $^{45}Ca^{2+}$. Ca^{2+} loss from the upper chamber was thus equivalent in all assays. (The normalized Scatchard plot for a given vesicle composition was independent of the concentration of vesicles in the assay (data not shown).) Binding constants for the family of curves generated by increasing the PC:cardiolipin ratio from 1:1 to 16:1 are summarized in Table 2. The apparent K_D of the higher affinity site increases monotonically as the mole fraction of cardiolipin in the vesicles is decreased. In contrast, the number of high-affinity Ca^{2+} binding sites per cardiolipin passes through a maximum at a PC/cardiolipin ratio of 4:1.

Adriamycin also inhibited the interaction of Ca^{2+} with cardiolipin-containing vesicles. Scatchard plots for flow dialysis measurements conducted in the presence of 20 μM adriamycin and 36 μM adriamycin are shown in Fig. 4. Table 3 compiles the binding constants for those plots, for measurements made at several other adriamycin concentrations using the same vesicle preparation (Experiment 1), and for several determinations with a second preparation (Experiment 2). The apparent number of high affinity Ca^{2+} binding sites was decreased markedly by the anthracycline antibiotic, with binding stoichiometry inversely related to drug concentration (r = −0.993 for Experiment 1, Table 3). The apparent K_D for the high-affinity interaction was, however, unaltered. Inhibition could not be attributed to disruption of

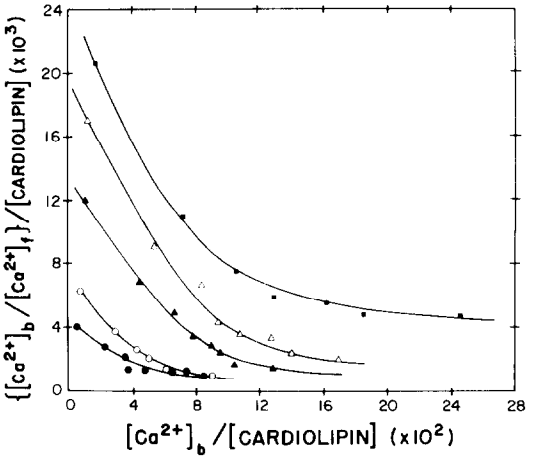

Fig. 3. Scatchard plots for Ca^{2+} binding to PC/cardiolipin vesicles of varied composition. All measurements utilized the protocol of Fig. 1. Vesicle concentration in the assay was adjusted so that all preparations bound similar amounts of Ca^{2+} (cpm/cpm_{max} = 0.44 ± 0.10 (S.D.) for fractions 11–13). The PC:cardiolipin mole ratios and the cardiolipin concentrations in the assays were, respectively, 2:1 and 79 μM (■), 4:1 and 118 μM (△), 8:1 and 130 μM (▲), 12:1 and 156 μM (○), and 16:1 and 180 μM (●). Data were analyzed as outlined in Fig. 1.

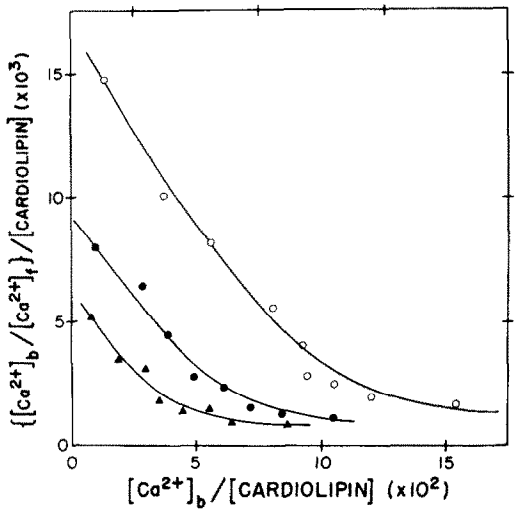

Fig. 4. Effect of adriamycin on Scatchard plots for the interaction of Ca^{2+} with cardiolipin-containing vesicles. The upper chamber contained PC/cardiolipin (4:1) vesicles equivalent to 95 μM cardiolipin. Adriamycin, when present, was added to the upper chamber prior to the vesicles: (○) no adriamycin; (●) 20 μM adriamycin; and (▲) 36 μM adriamycin. Similar results were obtained in a total of twelve runs with adriamycin concentrations between 20 and 48 μM. The protocol utilized was that of Fig. 1, except that, as outlined in Materials and Methods, buffer flow through the lower portion of the chamber was increased. This hastens the attainment of steady-state levels of $^{45}Ca^{2+}$ in the effluent stream and reduces loss of label from the upper chamber. Data analysis as for Fig. 1.

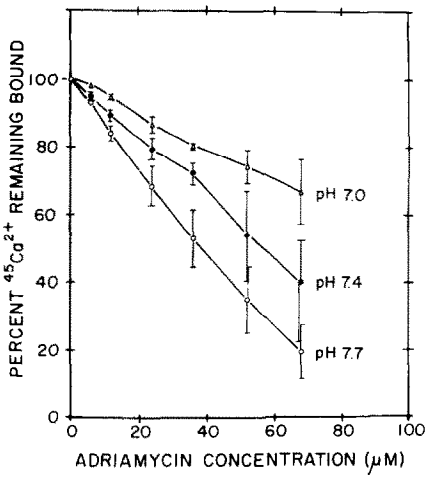

Fig. 5. Effect of pH on the ability of adriamycin to displace Ca^{2+} from PC/cardiolipin (4:1) vesicles. The experimental procedure was that outlined in the legend of Fig. 2. Results are shown for two series of vesicle preparations. The symbols indicate the average values, the bars the actual values, obtained.

the vesicles by adriamycin. No increase in the fluorescence of entrapped 6-carboxyfluorescein [43] was induced by the drug.

An attempt was made to assess the role of the adriamycin amino-radical in inhibition of Ca^{2+}-cardiolipin interaction in this system. Vesicles were formed and adriamycin effects subsequently determined at pH 7.0, 7.4, and 7.7. The pK_A of the amino group on adriamycin is reported to be *ca.* 8.0 [44]. An increase in pH from 7.0 to 7.7 would thus be predicted to decrease significantly the concentration of the charged form of the drug. In fact, an increase in pH increased the efficacy with which adriamycin was able to displace Ca^{2+} from cardiolipin-containing vesicles (Fig. 5). pH changes in this range had no detectable direct effect on Ca^{2+}–cardiolipin interaction (data not shown).

DISCUSSION

Unilamellar vesicles formed from PC and cardiolipin interacted with Ca^{2+} with apparent high affinity. The interaction can be attributed solely to the cardiolipin in the preparations since no Ca^{2+} binding by control vesicles prepared from PC only was detected (Fig. 1).

The Scatchard plots for the interaction of Ca^{2+} with the cardiolipin-containing vesicles are concave upward. Plots were analyzed according to Rosenthal [42] by assuming the presence of two independent classes of binding sites. (Alternate assumptions are possible.) Measurements of binding constants for

Table 3. Effect of adriamycin on binding constants for the interaction of Ca^{2+} with PC/cardiolipin (4:1) vesicles

Adriamycin concn (μM)	High-affinity site		Low-affinity site	
	K_D (μM)	n/CL	K_D (mM)	n/CL
Experiment 1				
	6.16	0.099	70.2	42.1
20	6.94	0.061	2.45	1.23
28	8.74	0.056	200.0	80.0
36	6.66	0.038	2.57	1.31
44	6.89	0.029	0.85	0.47
Experiment 2				
	5.47	0.106	28.2	17.5
28	5.83	0.068	10.1	5.1

Data were obtained as outlined in Fig. 4 and analyzed according to Rosenthal [42].

the assumed high-affinity binding site were highly reproducible: apparent $K_D = 5.57 \pm 0.48\ \mu M$; Ca^{2+} bound per cardiolipin $= 0.101 \pm 0.007$ (four vesicle preparations; PC:cardiolipin ratio = 4:1). Binding constants for the assumed low-affinity site varied much more widely. Some of the variability resulted from the flow dialysis protocol. Data used in estimating the constants for the low-affinity site were obtained late in any experiment. At that point, the relative change in free $^{45}Ca^{2+}$ concentration upon addition of non-radioactive Ca^{2+} was small (Fig. 1), and, thus, subject to enhanced error. In addition, during curve fitting, substantial changes in the binding constants of the low-affinity site resulted in relatively minor alterations in the shape of the resultant curve. It is clear that the data support the presence of a second binding component with a K_D for Ca^{2+} in the millimolar range. Further comments on that site, however, are unwarranted at present. The remainder of the discussion focuses on the high-affinity site.

Ca^{2+}-cardiolipin interaction was inhibited by adriamycin at concentrations as low as 10 μM (Fig. 5). Binding of adriamycin to cardiolipin has been reported to be primarily electrostatic in nature [45, 46], although the complex is stabilized by stacking interactions between the anthracycline rings of adriamycin molecules bound to the same cardiolipin [9]. Adriamycin substantially alters the apparent K_D for Ca^{2+}–cardiolipin interaction in a two-phase organic extraction system [35]. We therefore expected that adriamycin and Ca^{2+} would compete in this model for the negatively charged cardiolipin headgroup. The data in Figs. 4 and 5 and Table 3 run contrary to that expectation. Adriamycin decreased the stoichiometry of high-affinity Ca^{2+}-vesicle interaction, but the apparent K_D for the interaction was unaltered. Furthermore, the inhibitory potency of adriamycin was increased by an increase in pH from 7.0 to 7.7. Changes in pH in this range had little effect on the ionization of the phosphate groups of cardiolipin as evidenced by the absence of detectable changes in Ca^{2+} binding. It can therefore be proposed that the uncharged form of the adriamycin molecule inhibited more strongly than the conjugate acid in this model system, possibly as a result of hydrophobic interactions of the anthracycline ring with the bilayer.

A pK_A value of approximately 8.0 has been reported for adriamycin in dilute solution [44]. Although the pH of the bulk solution was varied between 7.0 and 7.7, in these experiments, it can be argued that the pH adjacent to the surface of the negatively charged, cardiolipin-containing vesicles was appreciably lower. In effect, all adriamycin in the region near the membrane would be in the aminoradical form. Alternative explanations for the effect of pH on inhibition of Ca^{2+}–vesicle interaction by adriamycin should therefore be considered. Pietronigro *et al.* [47] have reported that increased pH, in the physiological range, promotes the spontaneous formation of adriamycin free radicals. The experiments reported here were conducted in air. Peroxidation of vesicle lipids either by adriamycin free radicals or by activated oxygen species may thus have mediated the inhibition.

^{31}P-NMR measurements utilizing concentrated dispersions of cardiolipin have demonstrated that adriamycin prevents induction of the inverted hexagonal H_{II} phase by millimolar Ca^{2+} [48]. Whether the effects of the drug are direct or are mediated by lipid peroxidation, the inhibition of Ca^{2+} binding to the model membrane system examined here may reflect a similar disruption of inverted phase formation by adriamycin.

An increase in PC:cardiolipin ratio also inhibited the high-affinity interaction of Ca^{2+} with cardiolipin-containing vesicles (Table 2). The K_D for the interaction was increased, and, for PC:cardiolipin ratios greater than 4:1, the number of Ca^{2+} bound per cardiolipin was decreased. We have reported that, in a two-phase organic extraction system, PC decreases only the stoichiometry of Ca^{2+}-cardiolipin interaction [34]. We suggested that this inhibition, by a phospholipid reported to stabilize bilayers [49], implicates inverted structures in Ca^{2+}–cardiolipin interaction. In the liposome model system, an effect of PC:cardiolipin ratio on surface charge density and thence on apparent K_D would also be expected. Superimposition of these two effects may explain the failure of Serhan *et al.* [31, 32] to observe cardiolipin-mediated Ca^{2+} uptake in liposomes containing 5 mole percent cardiolipin or less.

The stoichiometry of high-affinity Ca^{2+} binding in these experiments approached a maximum value of 0.1 Ca^{2+}/cardiolipin for PC/cardiolipin (4:1) vesicles. It was increased somewhat less than 2-fold upon addition of A23187 which allows Ca^{2+} access to cardiolipin in the internal leaflet of the bilayer. This value is still low compared to the 1:1 binding stoichiometry reported for dispersions of cardiolipin in the presence of excess Ca^{2+} [27, 34]. Interaction of cardiolipin with Ca^{2+} may thus, not surprisingly, be constrained by incorporation of the lipid into a bilayer.

Attempts to increase the stoichiometry of high-affinity Ca^{2+} binding by decreasing the PC:cardiolipin ratio below 4:1 were not successful. Ca^{2+} binding to these vesicles was decreased markedly (Table 2). At the same time, for PC/cardiolipin 1:1 mixtures, vesicle yield after Sephadex G-50 gel filtration was reduced. Schiefer *et al.* [50] reported an inability to measure consistent ESR spectra for sonicated vesicles prepared from cardiolipin only. Vesicles with high cardiolipin content may, thus, differ qualitatively from vesicles containing higher mole fractions of PC. Neither phosphatidylethanolamine [37] nor monogalactosyldiglyceride [51], both of which spontaneously assume inverted configurations, will form vesicles.

If Ca^{2+}-cardiolipin interactions are to be considered physiologically meaningful, they must occur at cytosolic Ca^{2+} concentrations. The apparent K_D for the high-affinity interaction of cardiolipin-containing vesicles with Ca^{2+} determined in this study fell between 2.7 and 10.7 μM, depending on PC:cardiolipin ratio (Table 2). This value can be compared with the K_D determined from initial rate studies for the electrophoretic Ca^{2+} uptake system of mitochondria (2 μM; [33]) and with the K_D for Ca^{2+} of the mitochondrial Na^+/Ca^{2+}-exchange system (13 μM; [52]).

The extent to which PC/cardiolipin vesicles interacted with Ca^{2+} in this model system was a function both of vesicle concentration (Table 1) and of PC:cardiolipin ratio (Fig. 3 and Table 2). The upper portion of the flow dialysis chamber contained 50–200 nmoles/ml cardiolipin. For comparison, the cardiolipin content of heart and liver cells was estimated at 3.9 and 0.9 μmoles/g wet weight, respectively*, i.e. cellular cardiolipin levels are higher. The approximate mole ratio of PC : phosphatidylethanolamine (PE) : cardiolipin in mitochondrial membranes is 4:4:1, with PC in slight excess over PE [12]. The PC : cardiolipin ratios utilized here bracket this value.

The major finding in this study is that Ca^{2+}–cardiolipin interaction can be considered a potential intracellular site for the cardiotoxic action of adriamycin. Inhibition of Ca^{2+}–cardiolipin interaction was detected at 10 μM drug, a concentration that can be considered therapeutically relevant. Plasma adriamycin concentrations reach 5 μM, falling rapidly to 20 nM, following a single high dose [55]; tissue levels can exceed plasma levels by two orders of magnitude or more, remaining essentially unchanged for 50 hr [56].

Further extrapolation from model studies of the type reported here would be premature. PC/cardiolipin vesicles represent a simplified system. In the mitochondrial membrane, behavior of cardiolipin will be subject to modification due to the presence of other phospholipids and proteins. In addition, detection of high-affinity cardiolipin–Ca^{2+} interactions in a model system provides little insight into the role of such interactions in the functioning cell. The finding that the interaction of cardiolipin with cations was selective for Ca^{2+} and that it was extremely sensitive to ruthenium red and La^{3+}, both classical inhibitors of mitochondrial Ca^{2+} uptake [33], suggests that the possible role of cardiolipin in Ca^{2+} fluxes across mitochondrial membranes warrants further study. In addition, the possibility that Ca^{2+}–cardiolipin interactions serve to modify the function of protein components of the inner mitochondrial membrane [29] should be given serious consideration. Such cardiolipin-mediated regulation by Ca^{2+} has been reported for erythrocyte acetylcholinesterase [57].

Acknowledgements—This work was supported by awards from the Graduate School and the Frank C. Bressler Research Fund of the University of Maryland at Baltimore and by ACS Institutional Research Grant IN-174A. Financial support in the form of a salary to P.M.S. was provided by NIH Grant NS 12063, USAMRDC Contract DAMD 17-81-C-1279, and an American Cancer Society Junior Faculty Research Award. C.E.N. was supported in part by a Short-Term Research Training Award from the University of Maryland School of Medicine. The authors are indebted to Charles Tomlin for fabrication of the flow dialysis chamber, to Nancy Thomas for atomic absorption measurements, to Dr. David Burt for assistance with Scatchard plot analyses, and to Drs. E. X. Albuquerque and F. C. Kauffman for their encouragement and support.

* Cardiolipin concentration was estimated using the following values: Mitochondrial content (mg mitochondrial protein/g cell wet weight): heart, 100 mg/g [53]; liver, 50 mg/g [54]. Mitochondrial lipid content (mg lipid/mg mitochondrial protein): heart, 0.32 mg/mg; liver, 0.18 mg/mg [12]. Cardiolipin content (percent of total phospholipid by weight): heart, 20%; liver, 17.2% [12]. Tissue density: 1 g/ml. Cardiolipin molecular weight: 1500. Contribution of neutral lipids to the overall mitochondrial lipids: <9% [12].

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