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Adriamycin—a potent inhibitor of Ca^{2+}–cardiolipin interaction

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Adriamycin (doxorubicin) is a potent antineoplastic agent, effective against a broad spectrum of leukemias and solid tumors [1, 2]. The therapeutic utility of the drug is limited, however, by its cumulative, and apparently irreversible, cardiotoxicity [3, 4].

The oncolytic activity of adriamycin is believed to result from intercalation of the anthracycline ring into the DNA double helix and subsequent interference with DNA replication and/or transcription [1, 5], which may be enhanced by localized generation of activated oxygen species [6]. Cardiotoxicity has been correlated with disruption of mitochondrial function [7]. Ultrastructurally, myelin-like figures indicative of drug–lipid interaction are seen [8, 9], and it has been shown that adriamycin interacts stoichiometrically with acidic phospholipids [10]. Complexation with cardiolipin (diphosphatidylglycerol), a lipid almost totally restricted in eukaryotic cells to the inner mitochondrial membrane, is particularly strong: $K_a = 1.6 \times 10^6\ M^{-1}$ [11].

Involvement of cardiolipin in Ca^{2+} transport across the mitochondrial inner membrane has been proposed based on the finding that Ca^{2+} induces cardiolipin to adopt inverted, non-bilayer, configurations [12, 13] and that cardiolipin can mediate the extraction of Ca^{2+} from an aqueous to an organic phase [14, 15]. Goormaghtigh *et al.* [16] have demonstrated, using ^{31}P-NMR, that adriamycin prevents both phenomena and have, therefore, suggested that the cardiotoxic side effects of adriamycin may result from altered mitochondrial Ca^{2+} fluxes.

Investigations of lipid organization using ^{31}P-NMR are limited to the use of high (millimolar) lipid concentrations and similarly high concentrations of ligand. The physiological relevance of the observations can therefore be questioned. We have described recently a two-phase organic extraction system which utilizes micromolar concentrations of both Ca^{2+} and lipid [17], and have shown that cardiolipin interacts with Ca^{2+} selectively and with apparent high affinity (K_d = 1–4 μM). I report here that adriamycin concentrations of 10 μM or less eliminate Ca^{2+}–cardiolipin interaction in this system at cytosolic Ca^{2+} concentrations (0.1 to 10 μM; [18]), and that drug effectiveness appears to be enhanced by positive cooperativity.

Materials and methods

The two-phase organic extraction system was a modification of that previously described [17]. Cardiolipin (4.9 to 9.5 nmoles) was dissolved in 1.0 ml toluene. The 2.0 ml aqueous phase contained 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-KOH, pH 8.0; $^{45}CaCl_2$ (1–300 μM); and adriamycin–HCl (0–50 μM). The two phases were vortexed together for 1 min and then separated by low-speed centrifugation. Samples of the organic and aqueous phases, the latter collected by puncturing the polypropylene tubes, were analyzed by standard liquid scintillation counting techniques. The concentration of Ca^{2+} bound to cardiolipin ($[Ca^{2+}]_b$) was calculated from the amount of $^{45}Ca^{2+}$ in the organic phase, and the free Ca^{2+} concentration ($[Ca^{2+}]_f$) from $^{45}Ca^{2+}$ remaining in the aqueous phase. The proportion of the total Ca^{2+} recovered, calculated from the sum of $[Ca^{2+}]_b$ + $[Ca^{2+}]_f$, increased with increasing total Ca^{2+} concentration and accounted for more than 80% of the label added to the tubes for Ca^{2+} concentrations >2 μM. Interfacial accumulation of a lipid–Ca^{2+} complex was, therefore, not considered in analyzing the data. No Ca^{2+} could be detected in the organic phase in the absence of cardiolipin, nor did adriamycin mediate the extraction of Ca^{2+}. The 1 min vortexing time was sufficient to maximize Ca^{2+} extraction into the organic phase. The data shown are representative of at least three experiments and are corrected for the Ca^{2+} content of the Hepes buffer (0.58 μM) determined by atomic absorption spectrophotometry.

Bovine heart cardiolipin (Na^+-salt) was purchased from Sigma and $^{45}CaCl_2$ from New England Nuclear. Toluene was Photrex grade from J. T. Baker. Adriamycin–HCl was provided by Dr. N. Bachur, University of Maryland Cancer Research Center.

Results

The effects of adriamycin on cardiolipin-mediated extraction of Ca^{2+} from an aqueous phase into an organic (toluene) phase are summarized in Fig. 1 and Table 1. Toluene was selected as an organic phase analog to the interior of

a biological membrane because its dielectric constant at room temperature (2.4) approximates the values reported for fatty acids (linoleic acid, 2.71; oleic acid, 2.46; palmitic acid, 2.30; Ref. 19) and for the inner mitochondrial membrane (2.7; Ref. 20). In addition, as reported for organic phases containing chloroform [10, 21], no appreciable partitioning of adriamycin into the toluene phase occurred in the absence of cardiolipin.

Inhibition of cardiolipin-mediated Ca^{2+} extraction was detected at 2 μM adriamycin, and an increase of adriamycin concentration to 5 μM reduced Ca^{2+} extraction by 50% or more at all Ca^{2+} concentrations tested (Fig. 1). At 15 μM adriamycin, Ca^{2+} extraction from aqueous phases containing cytosolic Ca^{2+} concentrations (0.1 to 10 μM; [18]) was undetectable. Scatchard plot analyses of the data (Table 1) indicate that adriamycin both increased the apparent K_d for the interaction and decreased the stoichiometry of Ca^{2+} bound per cardiolipin, with the effects on the former quantity more marked in all experiments. This is in keeping with the observation that both adriamycin [22, 23] and Ca^{2+} [24] interact with the phosphate moieties of the cardiolipin headgroup. (Direct binding of Ca^{2+} to adriamycin is a sufficiently low affinity event [25] to be ignored in this context.)

Quantitation of the effectiveness of adriamycin as an inhibitor of Ca^{2+}–cardiolipin interaction in this system has proven difficult. In four experiments using varied amounts of cardiolipin and 50 μM Ca^{2+}, 3.2 ± 1.2 μM (S.D.) adriamycin inhibited Ca^{2+} extraction 50%. It is clear from Fig. 1 that adriamycin inhibited even more effectively at more nearly cytosolic Ca^{2+} concentrations. Dixon plots for the inhibition are concave upward (Fig. 2). Such deviation from linearity suggests that the inhibition was positively cooperative. This is consistent with the report that 2:1 (adriamycin/cardiolipin) complexes, formed as a result of electrostatic interactions between the amino-radical of adriamycin and the phosphate groups of cardiolipin [22, 23], are stabilized by stacking interactions between the ring systems of the adjacent adriamycin molecules [11]. Alternatively, some of the upward curvature may reflect a cardiolipin-dependent decrease in inhibitor concentration in the aqueous phase. The amounts of cardiolipin (5–10 nmoles) and adriamycin (0–100 nmoles) used in these assays were comparable. Thus, especially at low Ca^{2+} and adriamycin concentrations, a substantial fraction of the adriamycin may enter the organic phase in the form of a cardiolipin–adriamycin complex [10]. This consideration would also explain both the failure of 2 μM adriamycin to reduce Ca^{2+} binding more sharply at low Ca^{2+} concentrations (Fig. 1) and the resultant minor effect on the apparent K_d for Ca^{2+} obtained with 2 μM adriamycin (Table 1).

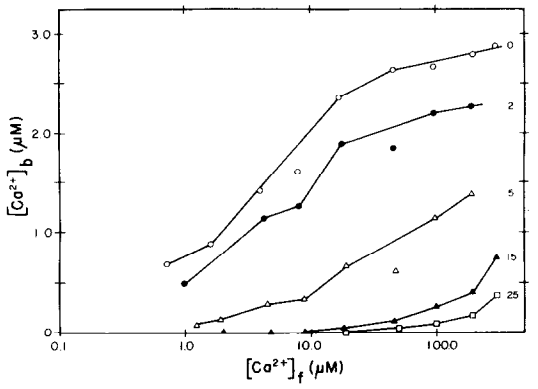

Fig. 1. Effect of adriamycin on Ca^{2+} binding to cardiolipin in a two-phase organic extraction system. Each tube contained 6.3 nmoles cardiolipin. Adriamycin concentrations (micromolar) are indicated by the numbers adjacent to the curves.

Table 1. Effect of adriamycin on the binding parameters for interaction of Ca^{2+} with cardiolipin*

Adriamycin concn (μM)	Apparent K_d (μM)	Ca^{2+}/Cardiolipin
0	4.1	0.45
2	5.1	0.36
5	20.6	0.21
10	45.0	0.08

* Binding parameters were derived from Scatchard plots constructed from data of the type shown in Fig. 1. The apparent K_d was calculated as the negative reciprocal of the slope. A value of 3.4 ± 0.9 μM (S.D.) was determined from four separate experiments in the absence of adriamycin. The stoichiometry of binding (Ca^{2+}/cardiolipin) was estimated from the intercept of the linear regression line with the x-axis.

Discussion

The ability of cardiolipin to interact, in a model system, with Ca^{2+} at concentrations found in the cytosol has been reported recently [17]. Data presented here demonstrate that the anthracycline antibiotic adriamycin severely inhibited that interaction. In this system, adriamycin effects were detected at drug concentrations two orders of magnitude below those required for inhibition of coenzyme Q-dependent enzymes [26], stimulation of production of activated oxygen species [27], or alteration of mitochondrial electron flow [28]. The data thus support the proposition that adriamycin–cardiolipin interaction may be involved in the cardiotoxicity of the drug, possibly by altering mitochondrial Ca^{2+} fluxes [16].

The ability of adriamycin to stimulate formation of activated oxygen species by mitochondria is well known (for a review see Ref. 29). If formation of an adriamycin–cardiolipin complex were followed by adriamycin-mediated

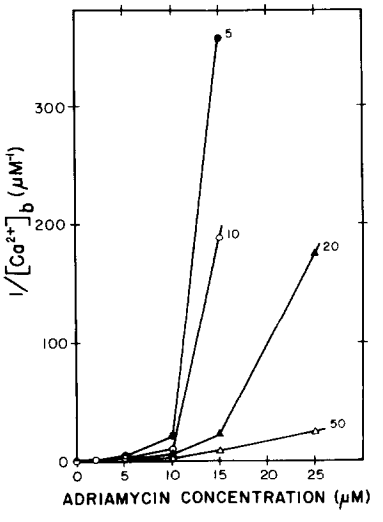

Fig. 2. Dixon plots for adriamycin inhibition of Ca^{2+}–cardiolipin interaction in a two-phase organic extraction system. Each tube contained 6.3 nmoles cardiolipin. Ca^{2+} concentrations (micromolar) are indicated by the numbers adjacent to the curves.

peroxidation of the highly unsaturated lipid [30], the consequent cellular damage would be both specific and irreversible.

In summary, adriamycin has been found to inhibit cardiolipin-mediated extraction of Ca^{2+} from an aqueous into an organic (toluene) phase. Inhibition of the Ca^{2+}–cardiolipin interaction was detected at 2 μM adriamycin, and 5 μM adriamycin reduced Ca^{2+} extraction >50% for all Ca^{2+} concentrations tested (1–300 μM). Adriamycin decreased the apparent affinity of cardiolipin for Ca^{2+}, with 10 μM adriamycin increasing the apparent K_d > 10-fold from the control value of 3.4 ± 0.9 μM (S.D.). Dixon plots suggest that the inhibition is positively cooperative.

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