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ANTIMITOTICS INDUCE CARDIOLIPIN CLUSTER FORMATION

POSSIBLE ROLE IN MITOCHONDRIAL ENZYME INACTIVATION

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We demonstrate here that drugs which inactivate cytochrome *c* oxidase are able to segregate cardiolipin essential for the enzyme activity, in a separate phase inaccessible for the enzyme. A molecular explanation of the drug-induced aggregation process is proposed.

There is at present, a general acceptance that several membrane enzymes require specific phospholipids for activity and that lipid fluidity mediates this activity. The effect of lipid fluidity has been assessed by reconstituting the enzyme environment with phospholipids of different fatty acid compositions. Kimelberg et al. [1] compared the discontinuities obtained for Arrhenius plots of enzyme activity $(Na^+ + K^+)$-ATPase reactivated with different phosphatidylglycerols and direct determination of their transition temperatures characterized by differential scanning calorimetry. Discontinuities appearing near the transition temperature of the phospholipids used, suggest strongly the essential role of fluidity on the enzyme activity. The observation that β-hydroxybutyrate dehydrogenase had a specific requirement for lecithin was among the first examples of lipid-dependent enzyme [2]. Subsequent to this work, several mitochondrial enzymes have been found to be lipid dependent after extraction of phospholipids by organic solvents, detergents or action of phospholipases. The absolute requirement of cardiolipin for the last oxidation site of the respiratory chain was definitively demonstrated by Fry [3], even if the number of cardiolipin molecules associated with the cytochrome *c* oxidase is still discussed.

We recently proposed that inhibition of the last oxidation site of the respiratory chain (cytochrome *c* oxidase, EC 1.9.3.1.) could participate in the adriamycin-induced mitochondrial toxicity [4]. It was suggested that the inhibition mechanism was due to a specific interaction between adriamycin and cardiolipin, a specific lipid of the inner mitochondrial membrane. Tentatively, we assumed that this drug-lipid complexation induced the exclusion of cytochrome *c* oxidase from its essential cardiolipin environment. We demonstrate here that the formation of cardiolipin clusters induced by adriamycin is a prerequisite to the inactivation process. Three antimitotics with a similar affinity for cardiolipin were shown to affect differently the cytochrome *c* oxidase activity measured on bovine heart mitochondria. This observation can be explained in terms of clusters formation resulting from the mode of association of the antimitotic-cardiolipin complex.

Cytochrome *c* oxidase activity was measured spectrophotometrically on bovine heart mitochondria. Activity changes were recorded after incubation of mitochondria with adriamycin,

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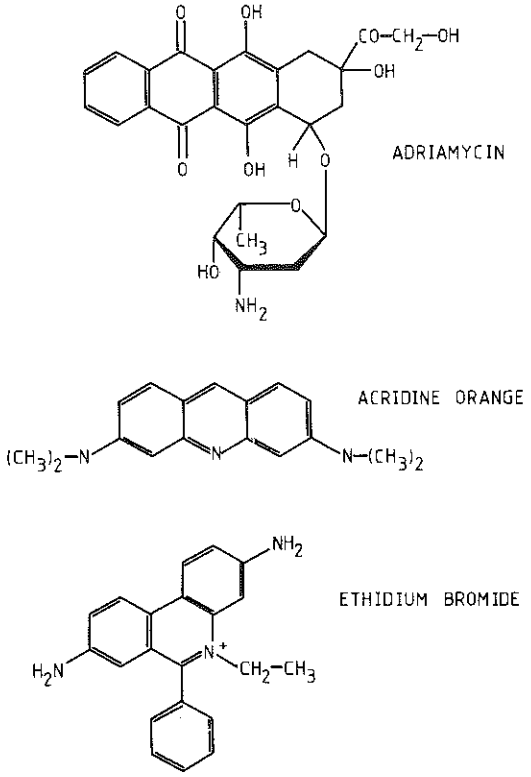

Fig. 1. Structure of the antimitotics.

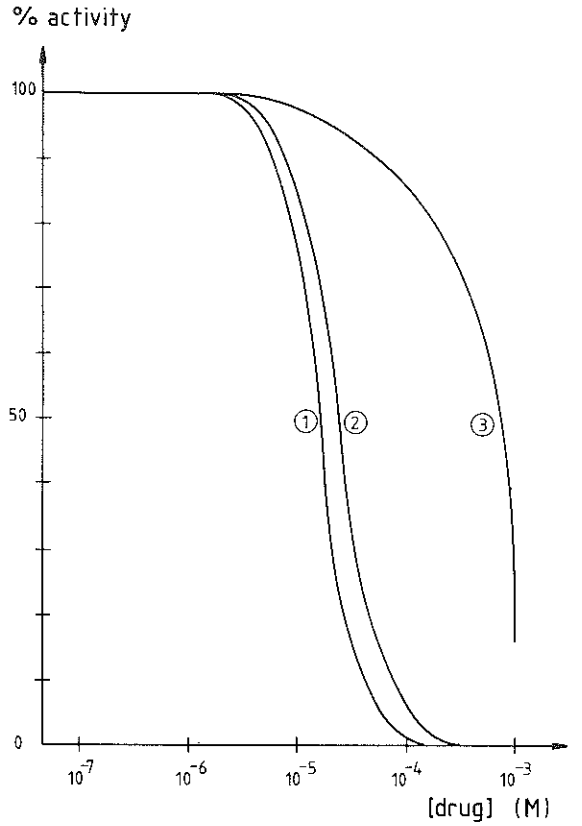

Fig. 2. Inhibition of cytochrome *c* oxidase activity. Freshly extracted bovine heart mitochondria following the method of Smith [9] were incubated 2 h in presence of (1) acridine orange; (2) adriamycin and (3) ethidium bromide. Reaction medium contained 0.030 mg of mitochondrial protein dissolved in 1.0 ml of buffer (10 mM Tris-HCl pH 7.4) containing deoxycholate (0.1%, v/v), the inhibitors of NADPH dehydrogenase (rotenone, 6 μg/cc) and of cytochrome *c* reductase (antimycin A_1, 10 μg/cc). The reaction was initiated by addition of 50 μl of reduced cytochrome *c* (final concentration of 56 μM). The cytochrome *c* is reduced by addition of dithionite (the excess of dithionite was eliminated by passage of reduced cytochrome *c*-dithionite mixture on a Sephadex PD-10 column). Initial rates of oxidation of cytochrome *c* were measured spectrophotometrically at 550 nm.

acridine orange and ethidium bromide (Fig. 1). The association constants of these three antimitotics with cardiolipin were evaluated using a procedure based on surface potential measurements and described elsewhere [5] (acridine orange-cardiolipin: $4 \cdot 10^6$ l/mol; adriamycin-cardiolipin: $2 \cdot 10^6$ l/mol; and ethidium bromide-cardiolipin: $2 \cdot 10^6$ l/mol).

Adriamycin and acridine orange inhibit 50% of the cytochrome *c* oxidase activity at concentration of $1.25 \cdot 10^{-5}$ M for acridine orange and $2.85 \cdot 10^{-5}$ M for adriamycin whereas a 10^{-3} M ethidium bromide concentration was needed (Fig. 2). The inhibition curves obtained with adriamycin and acridine orange may be explained in terms of slightly different association constants. The completely different behaviour observed with ethidium bromide cannot be explained in these terms. For adriamycin, it has been demonstrated that the cytochrome *c* oxidase inhibition resulted from the complexation of the enzyme cardiolipin environment rather from a drug-enzyme direct interaction [4]. In our experimental conditions, the antimitotic concentration (10^{-5} M) is at least ten times the cardiolipin concentration if one takes into account the cardiolipin proportion in the inner mitochondrial membrane.

Differential scanning calorimetry measurements (DSC) were performed to obtain a molecular pic-

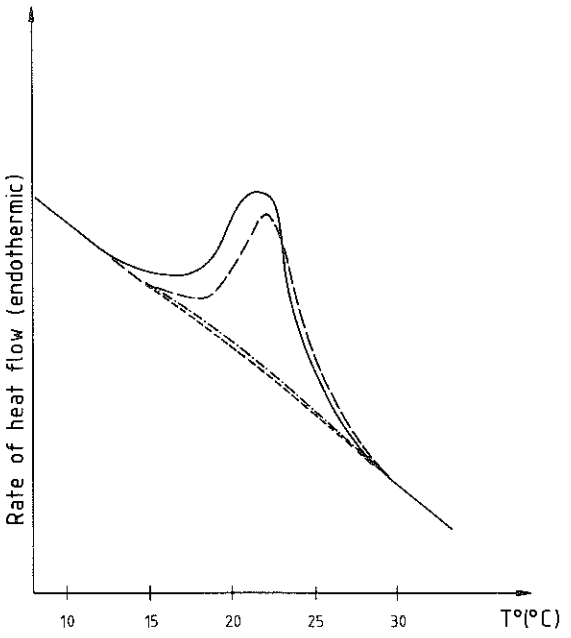

Fig. 3. Differential scanning calorimetry heating curves of DPPC-cardiolipin multilamellar liposomes in absence (- - - - - -) and in presence of antimitotic (adriamycin (— — —); acridine orange (———); ethidium bromide (– · – · –)). The liposomes are prepared as follows: DMPC (Sigma) is dissolved in chloroform, cardiolipin (Sigma) and antimitotics in ethanol. The solvents were evaporated under nitrogen flow and the lipid-drug film was dried overnight under vacuum. Liposomes were obtained by mechanical stirring (vortex mixer) of the drug lipid film in buffer (10 mM Tris-HCl, pH 7.4). The temperature was maintained above the lipid phase transition temperature (T_c) of the DMPC. The liposomes contained 70% (w/w) of DPPC (55 mM final concentration) and 30% (w/w) of cardiolipin (12 mM final concentration). When antimitotic is added, the final drug concentration is 24 mM (twice that of cardiolipin). For each spectra, 100 μl of solution were used. The reference is constituted by 100 μl of the buffer solution. Differential scanning calorimetry spectra were recorded on a SETARAM 111 using 100 μl per cell. The heating rate is 2°C/min. Before the measurement, the samples were left to stand at room temperature (20°C) for 6 h.

ture of the mode of organization of the antimitotic-cardiolipin complex in the lipid matrix. Results are given in Fig. 3. Multilamellar liposomes made of cardiolipin and dimyristoylphosphatidylcholine (DMPC) were formed and analyzed by DSC. The transition usually observed at 23.4°C with pure DMPC liposomes is abolished as a consequence of a modification of the DMPC-DMPC interactions induced by cardiolipin (Fig. 3). Addition of adriamycin or acridine orange to the multilamellar liposomes so formed restores the transition peak characterizing a pure DMPC phase (Fig. 3). Addition of ethidium bromide does not restore the transition peak characterizing a pure DMPC phase. This result indicates that adriamycin-cardiolipin and acridine orange-cardiolipin complex segregate in the lipid matrix whereas the ethidium bromide-cardiolipin complex is randomly distributed, suggesting that cardiolipin clustering plays a prominent role in the enzyme inactivation process.

To explain the cardiolipin clustering in terms of molecular interaction, the conformation of the antimitotic-lipid complex was analyzed using a procedure recently developed in our unit [6,7]. The total conformational energy is calculated as the sum of the contributions resulting from the Van der Waals interactions, electrostatic interactions, torsional potentials and transfer energy. To simulate the lipid-water interface, the electrostatic in-

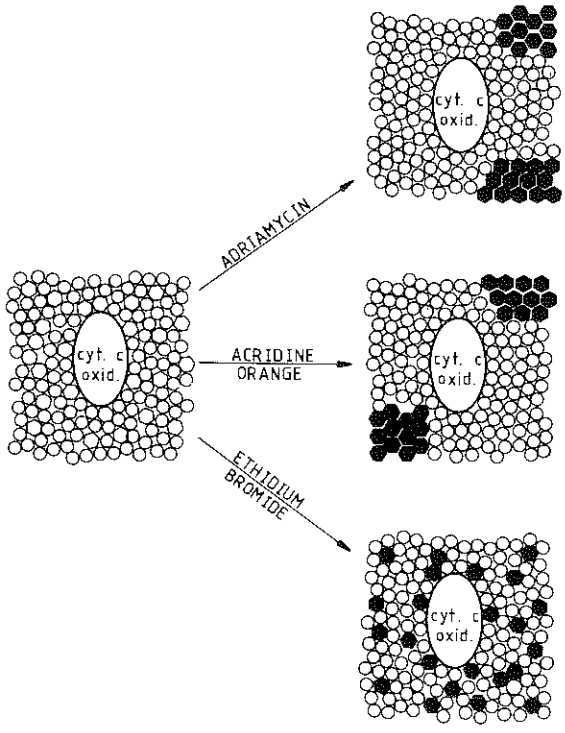

Fig. 4. Schematic representation of a possible enzyme inactivation mechanism. ○, Neutral lipid; ○, cardiolipin; ●, cardiolipin-antimitotic complex.

teraction was calculated for a dielectric constant of 3 and 30, respectively, in the hydrophobic and hydrophilic media [6,7].

A systemic study was first performed on the isolated cardiolipin molecule [7]. The resulting structure with a probability of existence of 89% was retained and submitted to a second analysis using the classical simplex minimization procedure [8]. Cardiolipin assemblage was obtained as described [7]. For 5 molecules, the total interaction energy between cardiolipin molecules (−27 kcal/mol) is largely in favour of an aggregate formation between lipids even if an electrostatic repulsion contributes to this value (+1 kcal/mol). In such a monolayer organization, the mean distance between phosphate is 6.3 Å; it corresponds to a mean area per molecule of 112 Å^2. If an identical procedure is applied to adriamycin, acridine orange and ethidium bromide molecules, we obtained monolayers structures with the following characteristics:

In the pure adriamycin monolayer, the total interaction energy (−3.88 kcal/mol) includes a Van der Waals contribution (−4.78 kcal/mol) enhanced by stacking between anthracycline rings; electrostatic interactions are slightly repulsive (0.90 kcal/mole). The area occupied per adriamycin molecule (55 Å^2) is almost half the area occupied per cardiolipin molecule (112 Å^2).

In the pure acridine orange monolayer, the mode of organization is similar to that observed for adriamycin (total interaction energy: −3.3 kcal/mol; area occupied per molecule: 50 Å^2).

In the pure ethidium bromide monolayer, even if the area occupied per molecule (60 Å^2) is similar to that of adriamycin, the total interaction energy (−0.13 kcal/mol) indicates the weak interactions between adjacent antimitotic molecules.

The low interaction energy observed between ethidium bromide molecules renders the cardiolipin clustering very improbable. On the other hand, the acridine orange-cardiolipin and adriamycin-cardiolipin complexes allow the formation of associated lipid structures stabilized by antimitotic-antimitotic interactions. The molecular structure of these 3 antimitotic-cardiolipin complexes will be described extensively elsewhere.

Finally, the present paper demonstrates the existence of a correlation between the capability of a drug to inactivate cytochrome *c* oxidase and to induce the formation of lipid clusters. A possible scheme of inactivation is proposed in Fig. 4. Only drugs capable of extracting cardiolipin from the lipid surrounding environment of cytochrome *c* oxidase and to form isolated lipid-drug aggregates can inactivate the enzyme. At the present time, the clustering has been only established in model membranes and an extrapolation to the mitochondrial membrane would be premature. For adriamycin, it is however tempting to design new structures avoiding the lipid cluster formation but preserving the interaction with DNA. It could be another possibility to decrease the antimitotic cardiotoxicity.

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