Sphingomyelin content conditions insertion of daunorubicin within phosphatidylcholine monolayers

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Abstract Cell death induced by the chemotherapeutic drug daunorubicin (DNR) implicates an apoptotic pathway originating at the plasma membrane and characterized by sphingomyelin (SM) hydrolysis and ceramide generation. The mechanisms by which such a drug (hypothetically passively diffusing across a structural membrane) can trigger SM hydrolysis is unknown, but raises the question of the precise interaction between DNR and membrane lipid constituents. In this initial study, using alternative current polarography together with voltammetry, we report that after a first step of adsorption, insertion of DNR within a condensed phosphatidylcholine monolayer was significantly facilitated by SM content.

Key words: Daunorubicin; Sphingomyelin; Lipid monolayer

1. Introduction

In previous studies, we demonstrated the involvement of the sphingomyelin (SM)–ceramide cycle in daunorubicin (DNR)-induced apoptosis [1]. It is clearly established that in most cells SM is found essentially within the outer leaflet of the plasma membrane. Present knowledge strongly suggests that under stress-induced apoptosis signaling, SM gains access to an intracellular sphingomyelinase (SMase) by flipping to the inner leaflet due to phospholipid (PL) scrambling [2]. Moreover, the absence of such a hydrolyzable SM pool within the plasma membrane inner leaflet has been associated with resistance to apoptosis [1]. It therefore appears that SMase activation is substrate dependent and that the spatial SM organization within the plasma membrane may be essential for the initiation of a SMase dependent apoptotic pathway.

Since DNR triggers the SM–ceramide cycle, which originates within the plasma membrane, this raises the question of how the initial process occurs. In order to better understand how DNR interacts with cell membranes and to investigate how it is affected by the presence of SM in the outer leaflet, we have studied the interaction of DNR with model membranes: phosphatidylcholine (PC) or PC/SM containing monolayers. Our study demonstrates that plasma membrane SM content directly facilitates DNR insertion within the PC containing monolayer through hydrophobic interactions.

2. Materials and methods

2.1. Drugs and chemicals

DNR (an anthracycline containing an amino sugar, daunosamine) was obtained from the National Cancer Institute Drug Repository and dissolved in water at a stock concentration of 1 mM. Chromatographically pure egg lecithin (PC grade I) and bovine brain SM were purchased from Lipid Products (Nutfield, UK) and supplied in chloroform–methanol solution. Mercury was purified and doubly distilled under vacuum. Ultrapure water was obtained from a Millipore Super Q system (Paris, France).

2.2. Preparation of monolayers

PL samples were evaporated in a stream of nitrogen; the lipid content was determined by weight and then samples were dissolved in hexane. A two-fold excess of lipid was spread over an aqueous solution, containing 0.15 M NaCl and 25 mM Tris at pH 7.4. This excess lipid formed collapsed structures in equilibrium with the spread monolayer and had a negligible contribution to the electrochemical measurements [3]. The excess was relative to the condensed state of PC obtained according to surface pressure experiments [3]. A condensed monolayer is in the nearest state of the outer layer of the biological membranes. Experiments were performed at room temperature, where the lipids are in the liquid–crystalline phase [4].

2.3. Electrochemical measurements

Alternative current (a.c.) polarography was carried out using the polarographic apparati as described previously [5]. The working electrode, a hanging mercury drop electrode (HMDE), was positioned in contact with the PL monolayer. An Ag/AgCl-saturated KCl electrode was the reference, and a platinum gauge was the auxiliary electrode. In order to obtain the differential capacity of the HMDE in direct contact with the monolayer, we measured the out of phase a.c. polarographic current. The electrical potential was imposed between the working and the reference electrodes. The potential was scanned at a rate of 50 mV/s, and the frequency of a.c. modulation (10 mV peak to peak) was 80 Hz. The starting potential was chosen in order to be in the stable region of the monolayer (−300 mV relative to the Ag/AgCl-saturated KCl electrode). Once the differential capacity was invariant, the equilibrium was reached. Analytical variations of the differential capacity were in the range of ~ 0.1 μF/cm^2.

Cyclic voltammograms were recorded between −0.2 V and −0.9 V at different sweep rates.

3. Results

3.1. Comparative differential capacity–potential curves of PC monolayers in the presence or not of SM

As previously reported [5], the a.c. polarographic method

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Abbreviations: DNR, daunorubicin; SM, sphingomyelin; SMase, sphingomyelinase; PC, phosphatidylcholine; PL, phospholipid; HMDE, hanging mercury drop electrode

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allows the study of the perturbations which can occur in a lipid monolayer by measuring the differential capacity variations of a Hg electrode in direct contact with a condensed PL monolayer while molecules, like proteins or drugs, are introduced into the solution beneath. This allows the detection of insertion of a hydrosoluble molecule within such a monolayer and its distinction from adsorption. A condensed monolayer is in the nearest state of the outer layer of the biological membranes. It has previously been demonstrated that a monolayer is formed under such conditions [6].

In Fig. 1, the differential capacity of the HMDE in direct contact with a PC or PC/SM containing monolayer was plotted versus the electrical potential. Three domains could be distinguished: (1) the PL monolayer was the most stable around the zero charge potential of mercury (−0.45 V) because of the weak interfacial electric field; (2) between −0.7 V and −0.9 V, with PC, two peaks appeared which were proposed to concern phase transitions of the layer [7]; (3) in the potential range more negative than −1.2 V, a desorption peak appeared. Indeed, in this range, due to high field effect, adsorption of cations from the electrolyte repels PL molecules adsorbed at the electrode.

In the presence of 30% SM, the second domain presented significant changes in the differential capacity. Indeed, the height of the two peaks decreased distinctly. This decrease indicated, from an electrochemical kinetic point of view, a slower process at the electrode. This could be ascribed to an increase in the stability of the layer by SM.

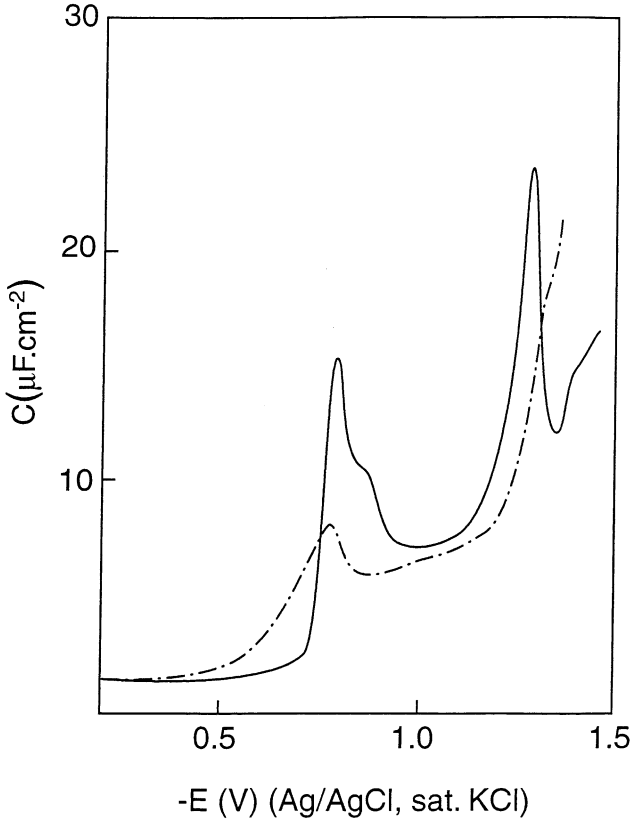

Fig. 1. Comparative differential capacity–potential curves of two PL monolayers. The differential capacity of the HMDE versus the electrical potential was measured by direct contact on PC monolayers in the absence (solid line) or in the presence of 30% SM (dashed line), as described in Section 2. Data shown are from a representative study.

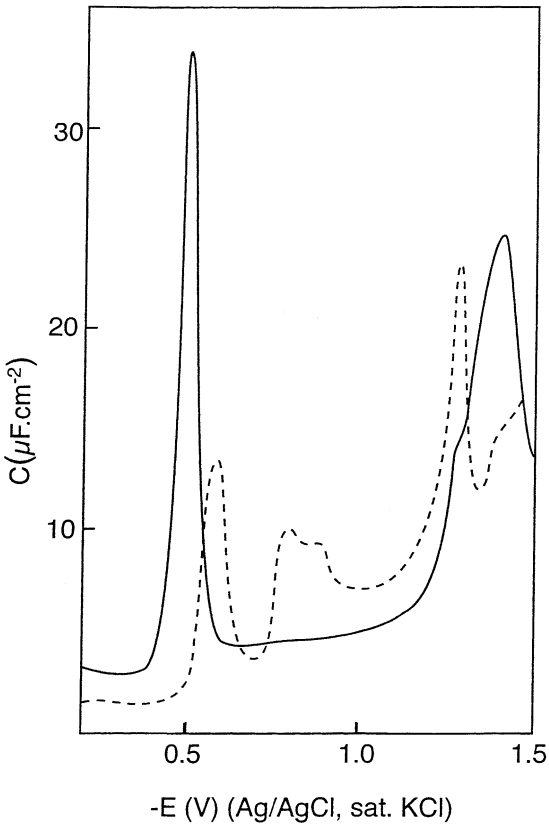

Fig. 2. Comparative differential capacity–potential curves of PL monolayers, in the presence of DNR. The differential capacity of the HMDE versus the electrical potential was measured by direct contact on PC monolayers in the absence (dashed line) or in the presence of 30% SM (solid line), after injection of 1 μM DNR, as described in Section 2. Data shown are from a representative study.

3.2. *Interaction of DNR with PC versus PC/SM containing monolayers*

In order to evaluate the effect of DNR on the PL monolayers, we measured the differential capacity versus the electrical potential after the injection of 1 μM DNR. As shown in Fig. 2, at about −0.3 V, a more positive potential than the zero charge potential, the differential capacity of the PC monolayer did not significantly increase, while it doubled in the monolayer containing 30% SM. This indicated a lack of significant insertion of DNR into a PC monolayer while it inserted with 30% SM, at the same DNR concentration.

Below 1 μM DNR, the constant differential capacity measured was not due to a lack of sensitivity of the method but rather to another mode of interaction of the drug with PC (described below) or to the absence of interaction.

Between −0.7 V and −0.9 V, the height of the peaks, observed with PC alone and with 30% SM (Fig. 1), decreased clearly in the presence of 1 μM DNR (Fig. 2). This observation meant that DNR interacted with the PL monolayers. Thus, on one hand, since DNR interacted with the monolayer (as shown at the −0.85 V peak) and as it did not insert with PC alone (as shown at −0.3 V), this indicated that DNR adsorbed at the PC/solution interface. On the other hand, the decrease of the peaks in the presence of DNR was more

pronounced at 30% SM (since the peaks disappeared totally) (Fig. 2), suggesting that DNR favored stabilization of both monolayers.

In the presence of DNR, one pseudo-capacitance peak appeared at −0.6 V with 100% PC and at −0.5 V with 30% SM (Fig. 2); the height was higher in the latter case. Taking into account the structure of DNR, the analysis of the reduction peak indicated that the potential of this peak was in the potential range of the reduction of the quinone [8].

3.3. SM effect on insertion of DNR into PC monolayers

In Fig. 3 are represented plots of the differential capacity of PL monolayers, composed of PC alone or of two SM contents relative to PC (at −0.3 V, just before the redox peak), as a function of DNR bulk concentration at equilibrium. Below a threshold concentration of DNR, depending on SM content, the differential capacity remained constant independently of DNR bulk concentration; we concluded that DNR adsorbs on the external boundary of the monolayer. Above this threshold concentration of DNR, the differential capacity increased indicative of an insertion process. It occurred around 2 μM DNR for the PC monolayer, 1 μM DNR in the presence of 15% SM, and at a much lower DNR concentration with 30% SM. Finally, a saturation of the process occurred above 0.5 μM DNR for 30% SM and 2 μM DNR for 0% SM as there was almost no further change in the differential capacity, with increasing drug concentration, reaching a similar value in the presence or not of SM. The differential capacity values are stable and reproducible below and above the DNR concentration threshold, while the steepness at the threshold is reproducible and depends on the lipid composition. The differences, concerning the DNR equilibrium threshold concentration, were due to a specific response of the layer to the drug in terms of insertion, which might be related to differences in affinity of the drug for the different monolayer compositions. The differential capacity is stable and reproducible below and above the threshold DNR concentration, while it is the steepness which is reproducible at the threshold, and it depends on the lipid composition.

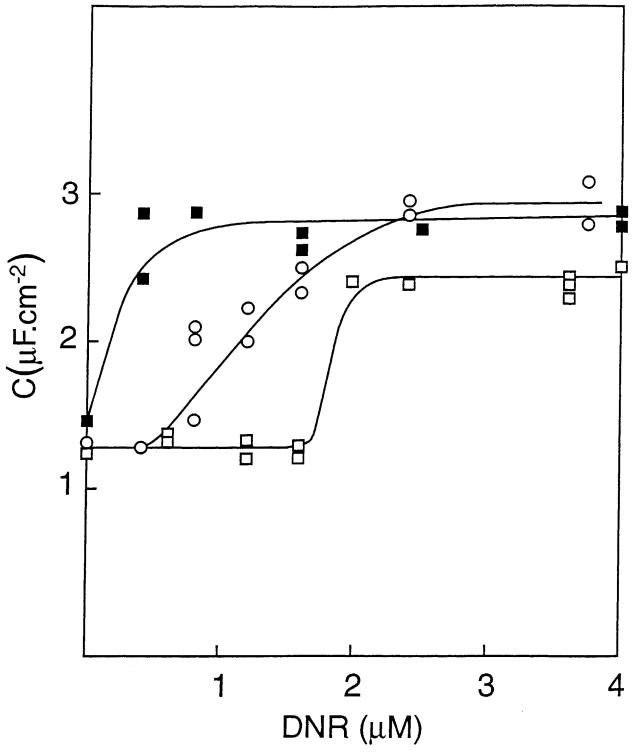

Fig. 3. Effects of SM and DNR on the differential capacity of PC monolayers. The differential capacity of the HMDE, at an electrical potential of −0.3 V (relative to a saturated Ag/AgCl electrode), was measured by direct contact with PC monolayers in the absence (□) and in the presence of 15% (○) and 30% (■) SM, after injection of different concentrations of DNR, as described in Section 2. Data shown are from a representative study.

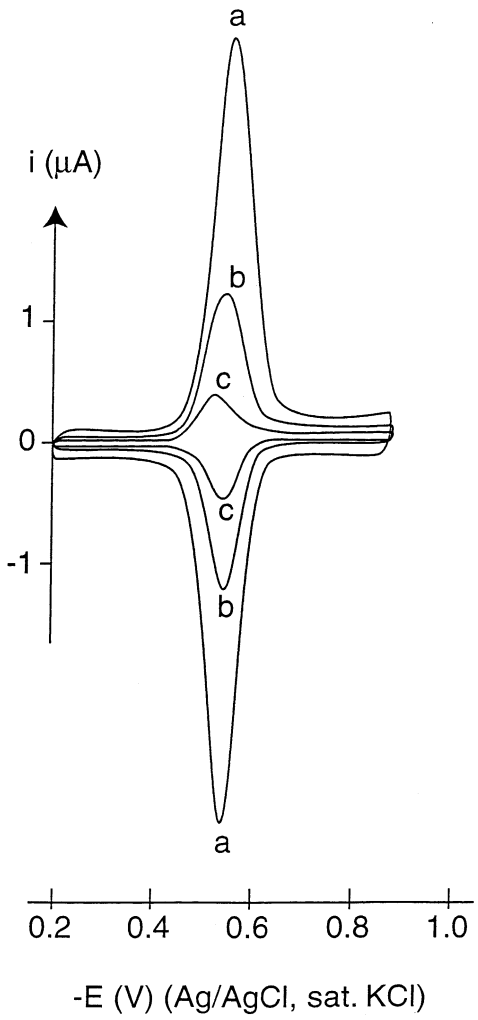

Fig. 4. Cyclic voltammograms at the HMDE in contact with a PC/30% SM containing monolayer, saturated with 2 μM DNR. The electrical current versus the electrical potential (relative to a saturated Ag/AgCl electrode) was measured by direct contact of the HMDE on PC/30% SM containing monolayer at high surface coverage of DNR, as described in Section 2. Initial potential: −0.2 V; sweeping of the potential range at: 0.5 V/s (a), 0.2 V/s (b), 0.1 V/s (c). Data shown are from a representative study.

3.4. Study of the redox system by voltammetry

Fig. 4 shows cyclic voltammograms at the HMDE in contact with a 70% PC/30% SM monolayer, saturated with DNR (2 μM in solution, see Fig. 3). Initially, the potential was held at −0.2 V and then cyclically swept between −0.2 V and −0.9 V at different sweep rates. The effect of the sweep rates was used in order to distinguish whether DNR was either confined at the monolayer (in contact with Hg) or reached the Hg drop

under diffusion control of the DNR molecules through the monolayer, during the electrochemical measurements. The reduction and oxidation peaks appeared at the same potential, which was indicative of a reversible redox process. It was observed that the voltammetric currents for the reversible peaks were proportional to the sweep rate. Consequently, it follows that the electric charges evolved under the peaks are constant and independent of the sweep rate. This corresponded to the amount of reduced/oxidized species, which was independent for the time the reaction was observed (sweep rates); this electrical signal indicated that the DNR molecules, containing the redox system, were confined at the monolayer in contact with Hg, and that there was no contribution to the electric charge arising from diffusing molecules. This is a classical criterion, in voltammetry, for a surface reaction. This confirmed the binding of DNR. The area of the peak allowed the calculation of the number of reduced quinones. For instance, at 30% SM, there was one reduced functional group per SM molecule.

4. Discussion

Previous reports, using different physical methods, circular dichroism or fluorescence resonance energy transfer have shown that the interaction between anthracyclines and PL (vesicles or membranes) was not proportional to the PL/drug ratio (whether evaluated in the absence or presence of DNA) [9,10]. Thus, different types of interactions were proposed to be involved, electrostatic and/or hydrophobic localized at the surface or within the membrane, respectively. While it has been proposed that DNR would remain in the middle of the head groups region or adsorbed on this part [11,12], there is no decisive evidence for these interpretations.

In order to address the question of how the binding occurs between DNR and SM containing PC monolayer, we used a.c. polarography. Using clinically relevant concentrations of DNR, it was possible, for the first time, to clearly detect distinct binding states, and more specifically to distinguish insertion from adsorption. Indeed, DNR at 0.5 μM adsorbed upon the 15% SM containing lipid monolayer, while insertion was only observed at 1 μM DNR (Fig. 3). The threshold value separating these two steps depended on SM content and decreased when SM content increased, indicating that SM favored DNR insertion and perhaps interaction within the lipid monolayer. Consistent information was obtained from the analysis of the redox peak potential by two ways. The voltammetric redox peak potential of DNR (Fig. 4) is not characteristic of the direct association of DNR with the Hg drop. It is only indicative of molecular species combined to the monolayer in contact with the Hg drop. Therefore, the peak potential might depend, to a certain extent, on the position of the DNR species relative to the monolayer (outside or inside). Indeed the pseudo-capacitance reduction peak potential of DNR (Fig. 2) shifted to more positive values in the presence of SM than in its absence, at a given DNR bulk concentration. This shift means that the DNR species are more easily reduced in the presence of SM. This must be the case for inserted species (in the presence of SM) compared to adsorbed species (in the absence of SM), since the hydration shell must be different in both cases. Since this redox peak corresponds to the quinone functional group, we may infer that DNR is inserted with the dihydroxyanthraquinone ring system, considered as hydrophobic. Concerning the adsorption, we propose that it could occur through the positively charged aminoglycosyl moiety of DNR (at pH 7.4) and the negatively charged phosphate group of PC or SM, in agreement with other interpretations [9].

Since SM is known to rigidify a fluid lipid layer [13], we propose that SM could stabilize the layer formed in the range of potential (between −0.7 V and −0.9 V), where we have observed a decrease of the differential capacity peaks. Furthermore, we propose for the same reasons that DNR tended to stabilize the monolayer as previously mentioned for bilayers [14].

In conclusion, our observations, obtained from the differential capacity measurements correlated with the voltammetric data, show a preferential insertion of DNR into SM containing monolayers. Our study is the first description of anthracycline interaction with a condensed SM containing PC monolayer, implicating two distinct binding steps. Hence, the existence of the DNR threshold concentration is evidence that cellular DNR accumulation is not a simple Fickian diffusion process. Furthermore, the interaction of DNR in membranes could affect their biological function and therefore the membrane may be considered as a potential target for the pharmacological action of DNR.

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