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Cholesterol Modulates Amiodarone-Membrane Interactions in Model and Native Membranes

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Abstract

The effects of cholesterol, a lipid mostly found in the sarcolemmal membranes, on the interaction of amiodarone with synthetic models of dimyristoylphosphatidylcholine (DMPC) and with native models of mitochondria and brain microsomes was studied. Alterations on the structural order of lipids were assessed by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) probing the bilayer core, and of the propionic acid derivative 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) probing the outer regions of the bilayer. As detected by the probes and according to classic observations, cholesterol progressively increased the molecular order in the fluid phase of DMPC. Additionally, it modulated the type and extension of amiodarone effects. For low cholesterol concentrations (≤10–15 mol%), amiodarone (50 μ*M*) ordered DMPC bilayers and the effects were almost identical to those observed in pure DMPC. For higher cholesterol concentrations, amiodarone ordering effects decreased slightly and faded for cholesterol concentrations as high as 25 and 30 mol%, when detected by DPH-PA and DPH, respectively. Above these high cholesterol concentrations, a crossover from ordering to disordering effects of amiodarone was apparent, either in the upper region of the bilayer or the hydrophobic core. The effects of amiodarone in native membranes of mitochondria and brain microsomes, in which "native" cholesterol accounts for about 0 and 25 mol%, respectively, correlated reasonably with the results in models of synthetic lipids. There is a close relationship between cholesterol concentration and

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amiodarone effects, in either synthetic models or native model membranes. Therefore, it may be predicted that the lipid physicochemical properties regulated by cholesterol concentration will also modulate the effects of amiodarone in sarcolemma.

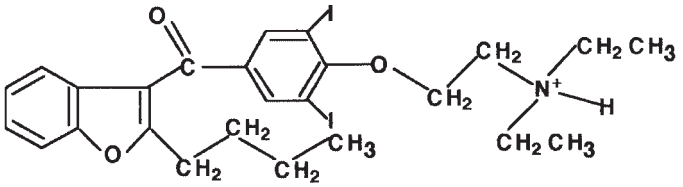
Index Entries: Amiodarone; membrane cholesterol; lipid physicochemical properties; membrane phases; fluorescent probes.

Introduction

Over the course of the past two decades, amiodarone (2-butyl-3-[3',5'diido-4'a-diethylaminoethoxybenzoyl]-benzofuran) has been widely used in the treatment of cardiac arrhythmias *(1–3)*. The antiarrhythmic activity of amiodarone, a prototype of Vaughan Williams class III agents *(4)*, results mainly in reducing ion-channel conductance, with K^+, Na^+, and Ca^{++} channels being the primary targets *(5,6)*. As a molecule with unparalleled efficacy and versatility, amiodarone is providing the background for the development of the ideal antiarrhythmic, if side actions can be favorably suppressed, from knowledge of structure-activity relationships *(2,3,6–10)*.

In spite of efforts in recent years, the molecular mechanisms underlying the antiarrhythmic action of amiodarone and those related to the undesirable effects are still poorly understood. The chemical properties of amiodarone *(3,11)*, its high partition into membranes *(12,13)*, and fundamental work *(14–27)* suggest a high affinity of amiodarone for membrane lipids. On the other hand, the activity of membrane-embedded proteins in general, and ion channels in particular, are regulated by the lipid physicochemical properties *(28–33)*. However, it is unclear which lipid membrane properties are critical for a given membrane function, and in the present study, for ion-channel activity. Therefore, our aim was to identify the matrix lipid properties driving the membrane-antiarrhythmic response of amiodarone.

Since native membranes are complex and difficult to handle and understand, the present work was started with phospholipid model systems reconstituted with lipids representative of heart sarcolemma. As a first approach, dimyristoylphosphatidylcholine (DMPC) was selected and enriched with increasing concentrations of cholesterol. Both lipids are well represented in sarcolemma, since phosphatidylcholine accounts for about 35% of the total phospholipids and cholesterol for about 37 mol% relatively to phospholipids *(34)*. The physical effects of amiodarone evaluated by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and the propionic acid derivative 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA), in the aforementioned synthetic model systems, were examined in parallel with perturbations in native model membranes of mitochondria and brain microsomes, in which cholesterol accounts for about 0 and 25 mol%, respectively *(35)*.



AMIODARONE
[2-butyl-3-(3', 5'diiodo-4'α-diethylamino ethoxybenzoyl)-benzofuran]

Fig. 1. Structure of amiodarone.

Materials and Methods

Materials

Amiodarone (Fig. 1) was provided by Sanofi-Labaz, Montpellier, France. The probes DPH and DPH-PA were purchased from Molecular Probes, Eugene, OR. DMPC and cholesterol were obtained from Sigma, St. Louis, MO. All the compounds were of the highest commercially available purity.

Preparation of Membranes for Fluorescence Polarization Studies

Synthetic model membranes were prepared as described elsewhere *(36)*. Briefly, solutions of pure DMPC in $CHCl_3$ were evaporated to dryness in round-bottomed flasks on a rotary evaporator. The resulting lipid film on the wall of a round-bottomed flask was hydrated with an appropriate volume of 50 m*M* KCl, 10 m*M* Tris-maleate, pH 7.0, and dispersed under N_2 atmosphere by hand shaking in a water bath set at 7–10°C above the transition temperature of DMPC. Then, multilamellar vesicles were obtained. Phospholipid-cholesterol bilayers were obtained by adding appropriate amounts of cholesterol to the phospholipid dissolved in $CHCl_3$. Native membranes, namely brain microsomes and rat liver mitochondria, were prepared as described elsewhere *(35)*. In all cases, the final nominal concentration of the lipid (phospholipid plus cholesterol) was 345 μ*M*. Model and native membranes were sonicated briefly and for a controlled period of time (about four bursts of 30 s each) to avoid the turbidity to decrease below 0.15 *A* units at 600 nm. This procedure does not distort the physical characteristics of multilamellar vesicles *(37)* but disperses aggregates and, consequently, decreases light scattering and improves the readings of fluorescence.

Incorporation of the Probes and Amiodarone into Liposomes

DPH and DPH-PA in dimethylformamide were injected (a few microliters) into membrane suspensions (345 μ*M* in total lipid) to give a final lipid/diphenylhexatriene probe molar ratio of about 300. The mixture was

initially vigorously vortexed for 10 s, and then amiodarone was added from concentrated ethanolic solutions. It was ascertained that added concentrations of amiodarone were within the solubility range. The mixture was incubated at 37°C in the dark to protect the probe for a period of 18–20 h to reach equilibrium, since the drug has to penetrate multiple bilayers. Control samples received equivalent volumes of dimethylformamide and ethanol. Added solvent volumes, always very small (a few microliters), had negligible effects on the measurements.

Fluorescence Polarization Measurements

Fluorescence polarizations were determined in a Perkin-Elmer spectrofluorometer, model MPF-3, provided with a thermostated cell holder. The excitation was set at 336 nm and the emission at 450 nm. The excitation and emission bandwidths were 6 and 8 nm, respectively. The temperature of the sample was checked with an accuracy of ±0.1°C, using a thermistor thermometer. The degree of fluorescence polarization (*P*) was calculated according to Shinitzky and Barenholz *(38)* from the following equation:

$$P = \frac{I_{\parallel} - (I_{\perp} \times G)}{I_{\parallel} + (I_{\perp} \times G)}$$

in which $I_{\parallel}$ is the intensity measured when the polarizer and analyzer are in the vertical position, and $I_{\perp}$ is the intensity when the analyzer filter is in the horizontal position. *G* is the grating correction factor for the optical system, given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction *(39)*. Depolarization effects as a consequence of scattering were taken into account and included in the grating factor measured at each temperature. All the fluorescence measurements were corrected for the contribution of light scattering by using controls with membranes, but without added probes. In our experimental conditions, the light scattering from the membrane vesicles was always very low (a maximum of 2% of the total signal obtained with the probes). However, the scattering value was always subtracted from the total fluorescence intensity of membranes labeled with the probes. Note that amiodarone, at concentrations used in the present work, has no effect on the fluorescence lifetime of the probes *(18)*.

DPH is a lipophilic probe known to incorporate in the hydrophobic core of the membrane and reports structural information in this region *(38)*. On the other hand, DPH-PA is anchored in close proximity to the bilayer surface by its propionate group, and the DPH moiety is embedded in the phospholipid acyl chains. Thus, this probe reports structural information on the bilayer lipid environments close to the surface, i.e., in the outer bilayer regions *(40)*. A high degree of polarization reflects a limited rotational diffusion of the probes and, therefore, reports a high structural order or low membrane fluidity and vice versa. The term *fluidity* is used here as

being inversely proportional to the degree of fluorescence polarization of DPH and DPH-PA probes and essentially reflects the rate of motion of phospholipid acyl chains. Note that only the fast lipid motion affects probe polarization, but not the tumbling of membrane vesicles, which is very slow compared with molecular motion *(41)*.

Results and Discussion

DMPC-Cholesterol Bilayers

A detailed study of DPH and DPH-PA fluorescence polarizations was carried out in DMPC bilayers with cholesterol concentrations ranging up to 50 mol% at 37°C (i.e., in the fluid phase of DMPC) in the absence and presence of 50 μ*M* amiodarone (Fig. 2). This concentration was selected on the basis of previous work *(21)*. As revealed by fluorescence polarization (*P*) of DPH and DPH-PA, and according to classic observations *(42,43)*, cholesterol progressively increases the molecular order in fluid DMPC bilayers (Fig. 2A,B, solid symbols). Additionally, very high cholesterol concentrations (50 mol%) endow the bilayer with fluorescence polarization values identical to those obtained in pure DMPC bilayers, in the gel phase *(21)*. Figure 2 also reports that cholesterol modulates the type and extension of amiodarone effects. Therefore, amiodarone induces ordering effects that are practically independent of cholesterol up to 10 or 15 mol%, as revealed by DPH-PA and DPH, respectively. Therefore, for these low cholesterol concentrations, the effects of the drug are almost identical to those observed in pure DMPC. Above these cholesterol concentrations, amiodarone ordering effects decrease slightly and fade for cholesterol concentrations of about 25 and 30 mol%, as detected by DPH-PA and DPH, respectively (Fig. 2A,B, open circles). Furthermore, a crossover from ordering to disordering effects is apparent above 25 mol% of cholesterol in the upper region of the bilayer, and above 30 mol% in the hydrophobic core.

The way by which cholesterol affects the physical state of lipids and, consequently, the interaction of amiodarone with the bilayer can be understood on the basis of the widely accepted phospholipid-cholesterol phase diagrams *(44–46)* or, in our experimental conditions, on the DMPC-cholesterol phase diagram *(47)*. Accordingly, in our system, at 37°C, two possible liquid phases may occur: a liquid-disordered (Ld), at low cholesterol concentrations (≤12.5 mol%), and a liquid-ordered (Lo), at high cholesterol concentrations (≥30 mol%). In the compositional range from about 12.5 to 30 mol% cholesterol, the phases Ld and Lo coexist. The Ld phase resembles the pure fluid lipid and Lo has intermediate properties between those of pure phospholipid fluid and gel; that is, it is a liquid (fluid) from the point of view of lateral disorder and diffusion but, at the same time, the phospholipid acyl chains are characterized by a high degree of conformational order *(45,46,48)*.

As illustrated in Fig. 2, for low cholesterol concentrations (≤10–15 mol%), amiodarone (50 μ*M*) induces ordering effects, which are identical to those

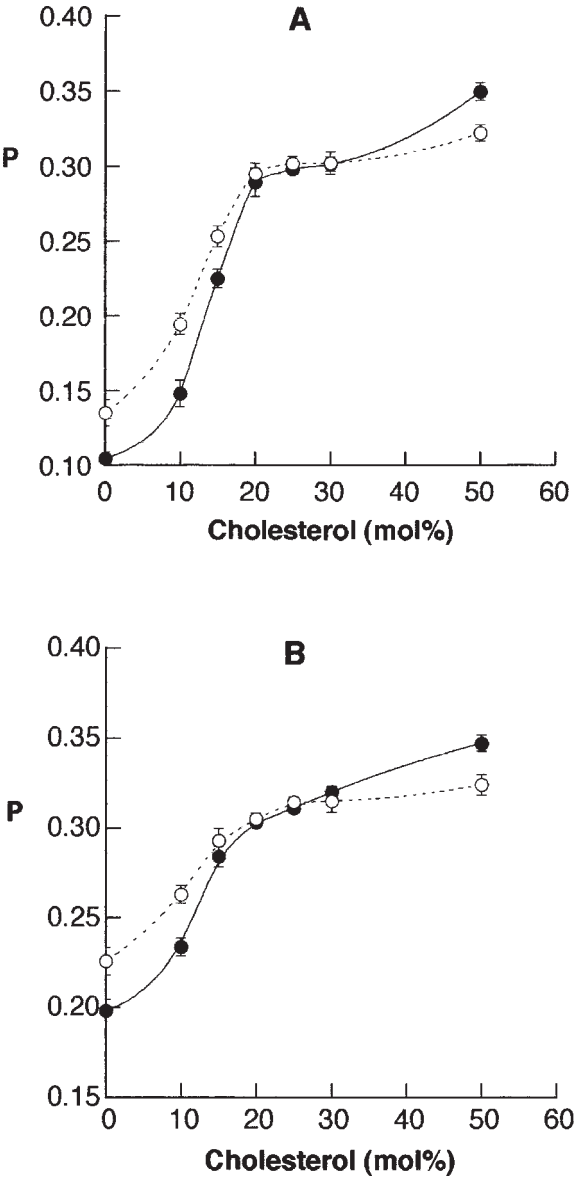


Fig. 2. Fluorescence polarization (*P*) of DPH **(A)** and DPH-PA **(B)** in DMPC bilayers as a function of cholesterol concentration in the absence (solid symbols) or presence (open symbols) of 50 μ*M* amiodarone at 37°C. Each point represents the mean ± SD from five to six independent experiments. Error bars for some data points are not represented, since they are encompassed by the size of the symbols.

induced in pure DMPC bilayers. At the temperature under study (37°C) and for these low cholesterol concentrations, the bilayer is, essentially, in the Ld phase. For cholesterol concentrations in the range of 10–15 to 30 mol%, at which the bilayer has two phase components, Ld and Lo,

amiodarone ordering effects decrease as cholesterol concentration increases and fade for cholesterol concentrations close to 30 mol% (i.e., when the bilayer is essentially composed of Lo phase). This means that amiodarone ordering effects are mainly related to the Ld phase, resulting in DMPC-amiodarone-rich domains separated from those induced by the packing of cholesterol with lipids in the cooperativity region *(49)*, creating the Lo phase. The results in Fig. 2 also indicate that for cholesterol concentrations higher than 30 mol%, at which the bilayer is in the Lo phase, characterized by a high degree of conformational order of phospholipid acyl chains, an apparent disordering effect of amiodarone is observed. This means that DMPC membranes enriched with cholesterol (≥30 mol%) do not prevent amiodarone interaction in accordance with studies revealing a reasonable partition even for very high cholesterol concentrations (unpublished results).

The discussed ordering and disordering effects of amiodarone, which are sensed across all the bilayer thickness, as revealed by DPH and DPH-PA, are closely related to the Ld and Lo phases, respectively, and can be tentatively explained as follows. According to previous data *(20–22)*, electrostatic and hydrophobic forces should determine amiodarone-membrane interactions. Additionally, studies in DMPC bilayers by Fourier transform infrared spectroscopy revealed that the electrostatic interactions take place in the carbonyl interface region of the bilayer *(22)*. Accordingly, the drug is localized in the intermediate region between the polar heads and the hydrocarbon tails of the phospholipids, which is consistent with the ionized form of amiodarone under the actual pH conditions *(15)*. Since amiodarone is not long enough to extend through the full length of the acyl chains, it creates voids that can be occupied by the phospholipid chains, and, consequently, interdigitation of two leaflets of the bilayer may occur *(22)*, explaining the ordering effects of amiodarone in Ld phase. On the other hand, the disordering effects of amiodarone in cholesterol-rich bilayers can be interpreted on the basis of their molecular geometries. According to Chatelain and Brasseur *(50)*, amiodarone was shown to be in the form of an inverted cone whereas cholesterol adopts a cone-shaped structure *(51)*. The association of the two cones results in a stable cylindrical structure, which, in turn, is stabilized when incorporated in the phosphatidylcholine matrix *(50)*. In the cylindrical structure, amiodarone and cholesterol should have a similar localization. Such a structure induces weakening of lipid-lipid interactions and an increase in disordering through the bilayer thickness, as detected by the probes DPH and DPH-PA. The fluidizing effects of amiodarone suggest that in its interaction with the sarcolemma, a hydrophobic mismatch between ion channels and surrounding lipids would take place.

The overall results of Fig. 2 together with previous data in the literature *(44–47)* indicate that the structural properties of different membrane phases, induced by cholesterol concentration, modulate the type and extension of amiodarone effects.

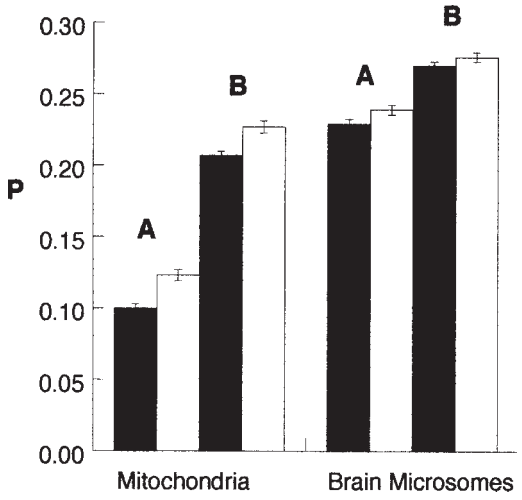


Fig. 3. Fluorescence polarization (*P*) of DPH **(A)** and DPH-PA **(B)** in native membranes of rat liver mitochondria and brain microsomes in the absence (solid bars) or presence (open bars) of 50 μ*M* amiodarone at 37°C. Each value represents the mean of five to six measurements ± SD. Cholesterol/phospholipid molar ratios for mitochondria and brain microsomes are about 0 and 25 mol%, respectively.

Native Membranes

The studies with amiodarone were also extended to native membranes with low and high cholesterol content attempting to collect pertinent information with biochemical significance. Therefore, representative native membranes, namely mitochondria and brain microsomes, differing in intrinsic cholesterol content, were chosen to study the physical effects of amiodarone, at 37°C (Fig. 3). As shown in Fig. 3 (solid bars), the fluorescence polarization of native membranes significantly depends on the intrinsic cholesterol content. Therefore, mitochondrial membranes with negligible content of cholesterol (≈0 mol%) are more fluid than those of brain microsomes, in which cholesterol accounts for about 25 mol%.

Regarding amiodarone effects (Fig. 3, open bars) the results correlate reasonably with those observed in models of synthetic lipids. Thus, apparent ordering effects of amiodarone are observed in cholesterol-poor membranes, such as mitochondria. On the other hand, the effects are very limited in brain microsomal membranes in which cholesterol accounts for about 25 mol%. Therefore, the ordering effects of amiodarone are more pronounced in mitochondria than in brain microsomes, as revealed by DPH and DPH-PA. Consequently, as in models of synthetic lipids, amiodarone effects in these native membranes are, certainly, conditioned by lipid physicochemical properties induced by cholesterol. Therefore, a future and more direct challenge is to search for a relationship between the lipid physicochemical properties induced by cholesterol and amiodarone effects, in native sarcolemma.

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