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Amiodarone Interactions with Membrane Lipids and with Growth of *Bacillus stearothermophilus* Used as a Model

Sónia M. L. J. Rosa, Maria C. Antunes-Madeira, Amália S. Jurado, and Vítor V. M. C. Madeira*

Centro de Neurociências, Departamento de Zoologia, Universidade de Coimbra, 3000 Coimbra, Portugal, E-mail: vmcm@ci.uc.pt

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Abstract

The thermophilic eubacterium *Bacillus stearothermophilus* was used as a model to study the effects of amiodarone (2-butyl-3-[3',5'diido-4'α-diethyl-aminoethoxybenzoyl]-benzofuran) in lipid organization and in bacterial growth. Effects 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. Amiodarone fluidizes bacterial polar lipid bilayers for temperatures below the phase transition midpoint, and orders the fluid phase of the bacterial polar lipids, as evaluated by DPH and DPH-PA. The ordering and disordering effects, which are concentration dependent, are more extensive when detected by DPH relative to DPH-PA. Growth studies performed in parallel revealed that amiodarone inhibits bacterial growth as a function of concentration. Amiodarone concentrations in the range from 1 to 2.5 μ*M* increased the lag time, decreased the specific growth rate, and decreased the final cell density. Furthermore, 3 μ*M* amiodarone completely inhibited growth. These in vivo effects of amiodarone can be related to its ability to perturb the phospholipid bilayer structure, whose integrity is essential for cell function, viability, and growth.

Index Entries: Amiodarone; *Bacillus stearothermophilus*; fluorescent probes; membrane organization; bacterial growth.

*Author to whom all correspondence and reprint requests should be addressed.

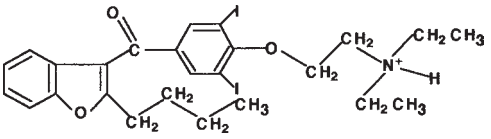

Fig. 1. Structure of amiodarone.

Introduction

Amiodarone (2-butyl-3-[3',5'diiodo-4'α-diethylaminoethoxybenzoyl]-benzofuran) has been widely used in the treatment of cardiac arrhythmias *(1,2)*. Unfortunately, the benefits of its use were accompanied by a number of side effects on the lungs, liver, nervous system, thyroid gland, cornea, and skin *(2)*, which may limit its use. In spite of research efforts in recent years, the molecular mechanisms underlying the antiarrhythmic action and those related with the undesirable effects are still not understood. The amphiphilic character of amiodarone (containing a large hydrophobic moiety and a small hydrophilic tail) (Fig. 1) and its low water solubility, 0.72 mg/mL *(3)*, suggest a high affinity of amiodarone for membranes. This idea is substantiated by the high partition coefficient of amiodarone into membranes, which is in the range of 10^4–10^6 *(4)*. This strong membrane incorporation leads to accumulation of amiodarone in the membrane, where it may perturb membrane organization and function. Previous studies indicate that amiodarone affects the physical state of lipids and membrane organization, in either models or native membranes *(5–9)*, and affects the membrane function *(10–16)*. However, in most studies of the past two decades amiodarone action proved extremely complex in complex potential membrane target sites. Consequently, interpretation of data is often difficult owing to putative multiple interactions that as well may be determined by basic membrane phenomena. Bacterial cells are often good models to study the action of membrane-active drugs *(17–24)* and to relate effects at the molecular level with physiological events. Most of the vital cell functions are ascribed to bacterial plasma membranes *(25–28)*, and, consequently, perturbations of membrane structure and function will impair viability and growth.

In the present study, *Bacillus stearothermophilus* was used as a model to study amiodarone-membrane interactions. In a first attempt, the effects of amiodarone on membrane physical properties were examined in parallel with perturbations of cell growth. Therefore, the aim of this work was to elucidate a putative relationship between changes in membrane organization and the physiological effects of amiodarone.

Materials and Methods

Chemicals

Amiodarone (2-butyl-3-[3',5'diiodo-4'α-diethylaminoethoxybenzoyl]-benzofuran) was provided by Sanofi-Labaz Research (Montpellier, France).

The probes 1,6-diphenyl-1,3,5-hexatriene (DPH) and 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid (DPH-PA) were purchased from Molecular Probes (Eugene, OR).

Growth Conditions

The strain of *B. stearothermophilus* used was isolated from discs impregnated with spores supplied by Mast (United Kingdom) (lot no. 7953). Conditions for its maintenance and growth have been previously described by Jurado et al. *(29)*. Liquid cultures were started with an early stationary phase inoculum from a complex medium (L-Broth) with a residual Ca^{2+} concentration of 115 μ*M* and were grown in 1-L Erlenmeyer flasks containing 200 mL of growth medium. The flasks were shaken at 130 strokes/min in a New Brunswick water bath shaker at 65°C. Amiodarone was added to the growth medium from a concentrated ethanolic solution in order to obtain concentrations ranging from 1 to 3 μ*M*. Growth was measured by turbidimetry, at 610 nm, in a Bausch & Lomb Spectronic 21 spectrophotometer.

Lipid Analysis

Growth was stopped at the beginning of the stationary phase by cooling the cultures on ice. Bacterial cells were harvested by centrifugation and washed three times with buffer (10 m*M* Tris-Cl, pH 7.0) and resuspended in the same buffer supplemented with 15 m*M* $MgCl_2$. Cells were converted to protoplasts by digestive action of 0.2% (w/v) lysozyme for 30 min at 37°C. Lipids were extracted by the method of Bligh and Dyer *(30)* and quantified by measuring the amount of inorganic phosphate *(31)* after hydrolysis of the extracts in 70% $HClO_4$ at 180°C *(32)*. The polar lipids were purified by preparative thin-layer chromatography on 2-mm thick silica gel plates (Merck, Darmstadt, Germany) developed in acetone to wash nonpolar components. The polar lipids remaining in the application zone were extracted with the mixture of solvents $CHCl_3$:CH_3OH:H_2O (45:45:10 [v/v/v]), followed by phase separation. The polar lipid phase was collected and evaporated to dryness on a rotary evaporator. The lipid residue was dissolved in a few milliliters of chloroform and stored under nitrogen atmosphere at –20°C.

Liposomes for Fluorescence Polarization Studies

Aliquots from bacterial polar lipid fraction in $CHCl_3$ containing 1.34 mg of lipid were evaporated to dryness on a rotary evaporator. The dry residues were hydrated under N_2 atmosphere at 50°C by gentle shaking with 5 mL of 50 m*M* KCl and 10 m*M* Tris-maleate (pH 7.0), and multilamellar vesicles were obtained. Then, the suspensions were vortexed for 1 min to disperse aggregates.

Incorporation of 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 55°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 *(33)* from the following equation:

$$P = [(I_{||} - I_{\perp}G)/(I_{||} + I_{\perp}G)]$$

in which $I_{||}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel ($||$) and perpendicular ($\perp$) to that of the exciting beam. The grating correction factor for the optical system (G) is given by the ratio of vertically to horizontally polarized emission components, when the excitation light is polarized in the horizontal plane *(34)*. 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. Amiodarone, at concentrations used in the present study, had no effect on the fluorescence lifetime of the probes *(11)*.

DPH is a lipophilic probe known to be localized in the hydrophobic core of the membrane and reports structural information in this region *(33)*. 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 *(35)*. 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, not the tumbling of membrane vesicles, which is very slow compared with molecular motion.

Results and Discussion

The effects of amiodarone on the physical organization of bacterial polar lipids, amounting to about 90% of the total lipids *(36)*, were studied. Fluorescence polarizations of DPH, a probe buried in the bilayer core *(33)*, and of DPH-PA, a probe anchored in the bilayer surface by its charged propionic group *(35)*, were examined in bacterial polar lipid dispersions to evaluate the relative perturbations induced by amiodarone on different regions across the membrane thickness.

Figure 2 displays the thermotropic profiles of bacterial polar lipid dispersions, evaluated by DPH and DPH-PA, in the absence (solid symbols) and presence (open symbols) of increasing concentrations of amiodarone (0–40 μ*M*). According to previous fluorescence polarization studies *(36)*, liposomes prepared with bacterial polar lipids display a broad phase transition, ranging from about 19 to about 33.5°C, with a midpoint (T_m) at 28 ± 0.5°C, as detected by DPH or DPH-PA (Fig. 2, solid symbols). It is clear from Fig. 2 that the range of polarization values of DPH-PA is wider compared with that of DPH, in agreement with the order of increase from the central to the outer bilayer regions *(37)*. Over the temperature range under study (10 to about 55°C), the thermotropic profiles of polar lipid bilayers were modified by the presence of 10, 20, and 40 μ*M* amiodarone. Disordering effects were observed for temperatures below the phase transition midpoint, which almost disappeared for temperatures of about 10°C (Fig. 2, open symbols). This may reflect the relative exclusion of amiodarone from the bilayer at low temperatures. Furthermore, the fluidizing effects, which are concentration dependent, were not identical along all the thickness of the bilayer. The region close to the hydrophobic core, detected by DPH, underwent larger perturbations. On the other hand, the derivative curves obtained from the fluorescence polarization data (Fig. 2, insets) show relative small effects in T_m accompanied by slight broadening effects of amiodarone relative to the control. However, an apparent shifting and broadening of the transition profile, promoted by amiodarone, has been detected by differential scanning calorimetry (DSC) data (unpublished results). Additionally, a shifting and broadening of the phase transition was also observed in models of synthetic lipids by fluorescence polarization *(5,7,8)*, by DSC *(38)*, and by Fourier transform infrared (FTIR) spectroscopy *(9)*. According to Jain and Wu *(39)*, the shifting and broadening of the transition profile suggest that amiodarone possibly localizes somewhere in the vicinity of the first eight carbons of the acyl chains, i.e., in the cooperativity region, which regulates, to a large extent, the sharpness of the thermal phase transition. Also, a position of amiodarone in the cooperativity region has been previously proposed by Chatelain et al. *(5)*.

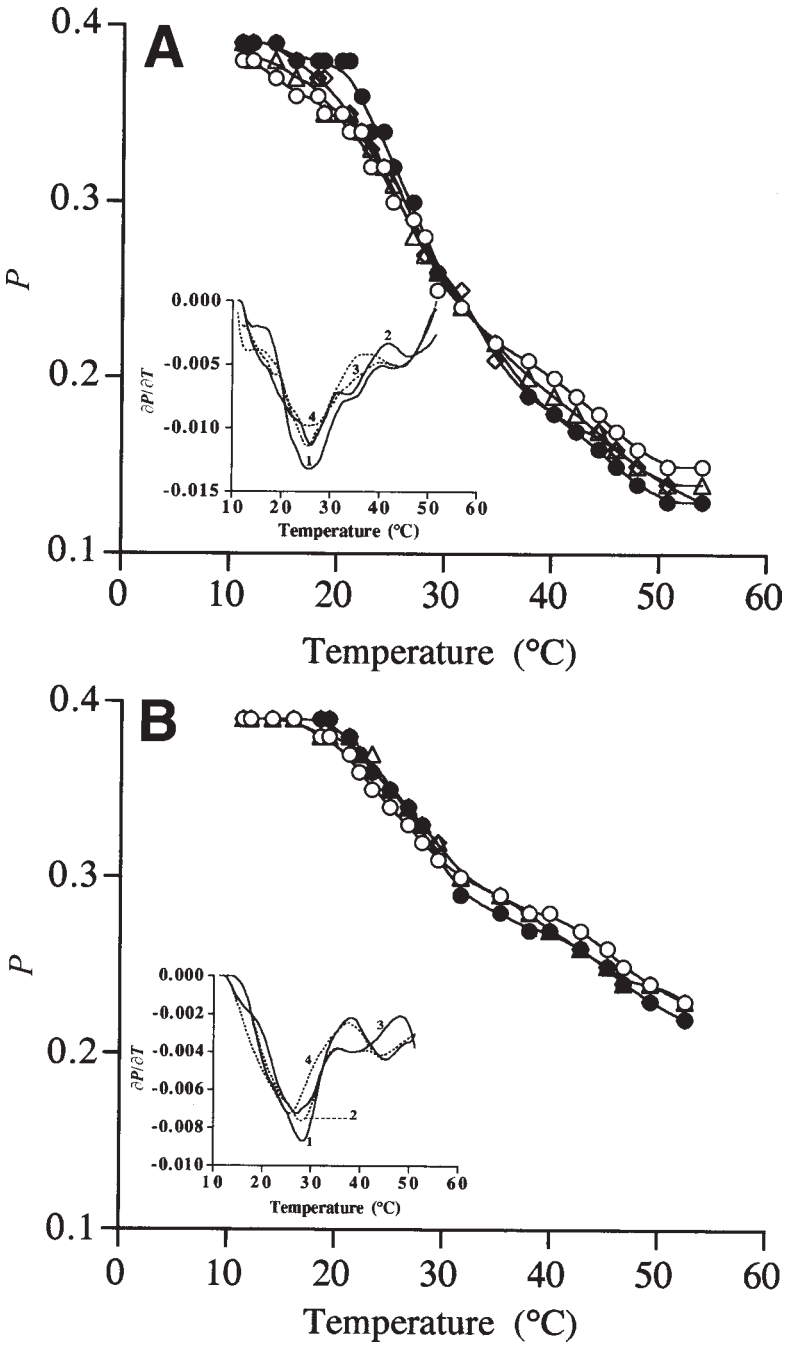

Fig. 2. Fluorescence polarization (*P*) of **(A)** DPH and **(B)** DPH-PA, in bacterial polar lipid bilayers, in the absence (solid symbols) or in the presence (open symbols) of increasing concentrations of amiodarone (◇, 10 μ*M*; △, 20 μ*M*; ○, 40 μ*M*). Insets represent the first derivatives of polarization data of the main plots. Derivative curves 1, 2, 3, and 4 correspond to the main curves with the symbols ●, ◇, △, and ○, respectively. The standard deviations (SDs) of the average of three experimental measurements, for each temperature, are too small to be displayed by error bars.

The data in Fig. 2 also indicate that amiodarone increases the fluorescence polarization in the fluid phase of bacterial polar lipids (temperatures above T_m) as a function of concentration. These ordering effects, more apparent when detected with DPH, are consistent with previous results obtained in models of synthetic lipids *(5,7,8)* and in fluid native membranes *(8)*.

The tertiary amine group of amiodarone has a pK_a of 8.7 *(5)* and, under the studied pH conditions (7.0), remains protonated. Therefore, the presence of amphiphilic amiodarone in the bilayer is expected to largely affect the equilibrium between the electrostatic interactions of the polar heads and the hydrophobic interactions of the acyl chains of phospholipids, because electrostatic and hydrophobic forces should determine amiodarone-membrane interactions according to previous data *(7–9)*. Additionally, Attal et al. *(9)*, who studied the interaction of amiodarone with dimyristoylphosphatidylcholine bilayers by FTIR spectroscopy, concluded that the electrostatic interactions take place in the carbonyl interface region of the bilayer. Accordingly, the drug is localized in the intermediate region between the polar heads and the hydrocarbon tails of the phospholipids, which is consistent with its amphiphilic character. Because 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, explaining the ordering effects of amiodarone in the fluid phase *(9)*.

The data in Fig. 2 also suggest that the extent of hydrophobic and electrostatic interactions is modulated by the physical state of the bilayer and, consequently, by the degree of hydration at the aqueous interface. Thus, the low degree of hydration in the gel phase *(40)* associated with the physical properties of the bilayer *(41)* would affect the conformation of the drug in such a way that the charge may be masked, as previously discussed *(8)*. A rotation in the single carbon-carbon bond of the ethoxy group of the drug may shorten the distance between the tertiary amine and the oxygen of the carbonyl group, and, consequently, an intramolecular hydrogen bond may take place. Therefore, the positive charge of the tertiary amine will suffer delocalization in this new geometry of amiodarone. As a result, the interaction of the drug with lipids will turn hydrophobic. In this condition, the phospholipid chains must distort to accommodate amiodarone, with weakening of lipid-lipid interactions, resulting in a disordering increase through the bilayer thickness, as detected by both the DPH and DPH-PA probes. On the other hand, in the fluid phase, the higher degree of hydration of phospholipid head groups *(40)* and the increased stability of the fluid phase favors electrostatic interactions and consequent ordering effects.

The ordering and disordering effects induced by amiodarone in polar lipids of *B. stearothermophilus* are essentially similar to the effects detected in synthetic membranes and native membranes of eukaryotics. Therefore, we are confident that the bacterial model system is suitable to generate reliable data underlying the membrane effects of amiodarone and implica-

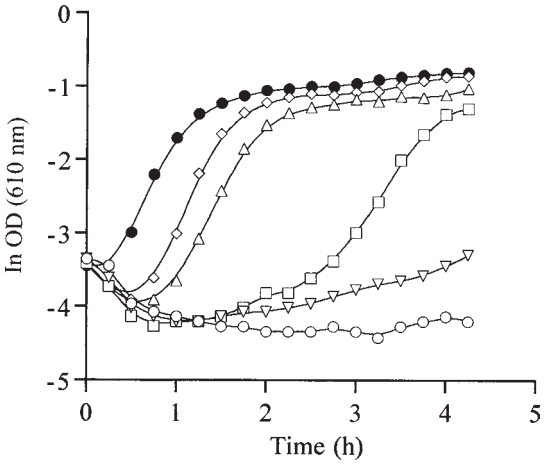

Fig. 3. Growth of cultures of *B. stearothermophilus*, at 65°C, in a complex medium (L-Broth), in the absence of amiodarone (control medium, ●) and in the presence of increasing concentrations (◇, 1 μ*M*; △, 1.5 μ*M*; □, 2 μ*M*; ▽, 2.5 μ*M*; ○, 3 μ*M*) of amiodarone. Growth was measured as optical density (OD) at 610 nm. As in Fig. 2, SDs of the experimental points were too small to be included.

tions in physiological activities, in an attempt to correlate in vivo and in vitro data.

In parallel with the physical studies, the effects of amiodarone on bacterial growth were studied. *B. stearothermophilus* was grown at 65°C (i.e., optimal temperature) in a complex medium (L-Broth) supplemented with amiodarone to obtain concentrations ranging from 1 to 3 μ*M*. It was shown that bacterial growth is impaired as amiodarone concentration is increased. The inhibitory effect of amiodarone was reflected by an increase in the lag time, a decrease in the specific growth rate, and a decreased final cell density (Figs. 3 and 4). Alteration of the growth parameters is a function of the drug concentration, and at 3 μ*M* amiodarone induced a complete cessation of growth (Fig. 3). According to previous work *(36)*, the growth of *B. stearothermophilus* is strongly affected by environmental conditions, ion concentration, and temperature parameters that affect the physical state of membrane lipids. Consequently, the effects of amiodarone on bacterial growth are certainly related to membrane effects, namely, through the interaction with lipids.

Conclusion

Amiodarone is likely to be located in the membrane along the cooperativity region and would extend to the bilayer surface (i.e., the carbonyl interface), establishing electrostatic and hydrophobic interactions with membrane components. The extent of hydrophobic and electrostatic interactions depends on the exact position of the drug and its geometry, which are modulated by the physical state of the bilayer and the degree of

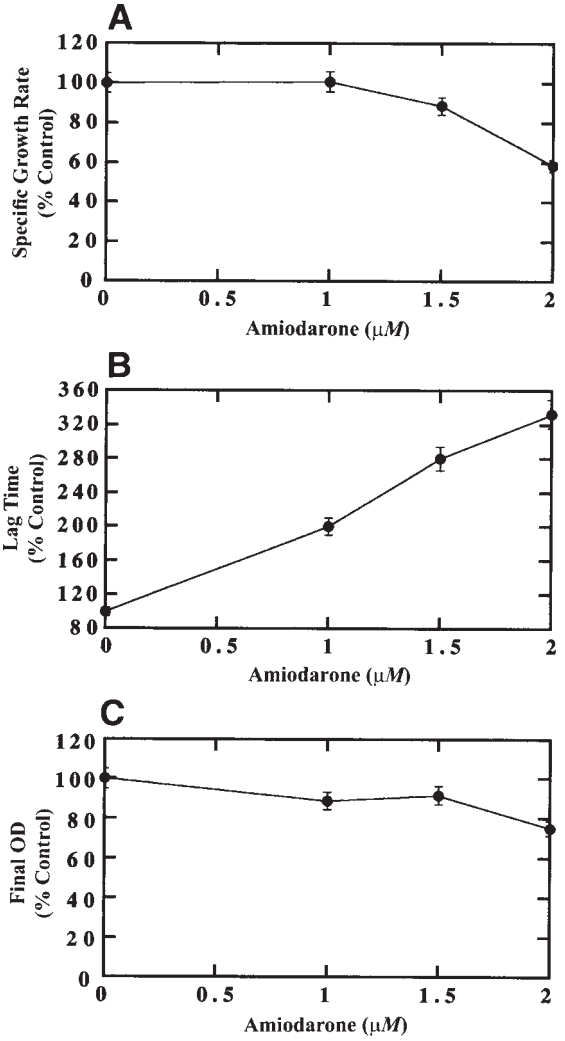

Fig. 4. Effects of increasing concentrations of amiodarone in **(A)** the lag time, **(B)** in the specific growth rate, and **(C)** in the final OD of cultures of *B. stearothermophilus* grown at 65°C. Data of Fig. 4 were taken from Fig. 3.

hydration at the aqueous interface. Therefore, the predominance of hydrophobic forces for temperatures below the phase transition midpoint of polar lipids induces disordering effects. On the other hand, in the fluid state (i.e., for temperatures above the phase transition midpoint) electrostatic interactions also occur and would contribute to the ordering effects of amiodarone. Therefore, amiodarone has specific effects on the structural properties of different membrane phases. These changes in fluidity will likely disturb membrane functions reflected in bacterial growth impairment, because the preservation of an exact degree of fluidity is essential for most membrane activities.

Acknowledgment

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