Biochemical Pharmacology, Vol. 36, No. 24, pp. 4245–4250, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

IONIZATION STATE OF AMIODARONE MEDIATES ITS MODE OF INTERACTION WITH LIPID BILAYERS

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(*Received* 27 *March* 1987; *accepted* 24 *June* 1987)

Abstract—Amiodarone is a potent antianginal and antiarrhythmic drug which affects the lipid dynamics. The influence of amiodarone ionization on the lipid transition temperature and enthalpy associated to the liquid crystalline to gel state transition was studied in multilamellar vesicles (MLV) of dipalmitoylphosphatidylcholine (DPPC) by differential scanning measurements (DSC) at different pH. These data were correlated with the calculated number of charged amiodarone molecules inserted into the lipid vesicles. The procedure of calculation requires the knowledge of the intrinsic ionization constant of amiodarone and the area occupied per amiodarone molecule in the close packed state; it can be applied successfully to water insoluble amphiphilic molecules. Only the ionized form of amiodarone molecule destabilizes the lipid matrix organisation whereas no effect was observed with the uncharged form. This destabilizing effect could be explained in terms of a modification of the drug structure induced by its ionization state or in terms of its distribution in the lipid matrix, as an isolated molecule or assembled in clusters depending on its ionization state.

Numerous agents with local anesthetic properties show antiarrhythmic properties in appropriate circumstances [1]. Specific interactions of the drug with membrane proteins implicated in ionic transport and exchange may account for these antiarrhythmic effects. However, because of the broad spectrum of chemical structures that can block excitation unspecific effects mediated by insertion of the agent into the lipid bilayer are often put forward [2]. The ionization state of an antiarrhythmic agent plays an essential role as it modulates the drug effects on the action potential [3, 4] through either direct interactions with the membrane proteins implicated in ionic transport [3] or modifications of the interaction with the lipid components of the membrane [4]. These modifications include variations of lipid solubility and electrostatic interactions with acidic phospholipids which are implicated in the Ca^{2+} homeostase and in the excitation–contraction coupling [5].

Amiodarone is a potent antiarrhythmic drug [6, 7]; its mode of action is poorly understood. Its effect on the physical state of membrane phospholipids has been investigated in multilamellar vesicles [8] and in natural membranes [9]. Amiodarone reduces the temperature of the gel to liquid crystalline phase transition and either increases or decreases lipid mobility in the gel or liquid crystalline phase [8]. The decrease in lipid mobility in the fluid phase is also observed in natural membranes with a threshold concentration of 10^{-6} M [9]. The inhibiting effect of amiodarone on the Na^+–K^+-ATPase has been attributed, at least in part, to the effect of the drug on the lipid dynamics [9]. Data from the literature [10–12] indicate that effective treatment of arrhythmias in the human is achieved for a plasma concentration of 1.50–3.80 10^{-6} M. Animal studies have shown that within the same range of drug concentration amiodarone is concentrated 10 times in the heart [13]. Finally, it was shown recently that a chronic treatment is able to decrease lipid mobility in erythrocytes ghost [14] and that amiodarone is present and concentrated in purified sarcolemmal membrane [15].

In the present communication, differential scanning calorimetry (DSC) measurements were performed with dipalmitoylphosphatidylcholine (DPPC) multilamellar vesicles in the presence of various amounts of amiodarone and at different pH. The lipid destabilization was analyzed in terms of the ionization state.

MATERIALS AND METHODS

Samples of amiodarone (2-butyl-3[4-(2-diethylamino-ethoxy)-3,5-diiodo-benzoyl]-benzofurane) were purchased from LABAZ-SANOFI. DL-α-Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chem. Co. (St. Louis, MO). The ionic strength of buffers (Tris/HCl, acetate/acetic acid, hydrogenocarbonate/NaOH) was kept constant (0.15 M NaCl). All reagents and solvents were pro analysis grade. The pH of the solutions was measured with a radiometer pH meter. The precision in the pH measurements was ±0.05 pH. Samples of multilamellar liposomes (MLV) were prepared by dissolving the lipid or the amiodarone–lipid mixture in chloroform. Chloroform was then evaporated to dryness under streams of N_2 and under vacuum. Buffer of appropriate pH was added and the mixture was shaken on a vortex mixer for 2–3 min above the transition temperature (Tr). The transition temperatures of the phospholipid dispersions were determined with a SETARAM DSC (type III, Lyon,

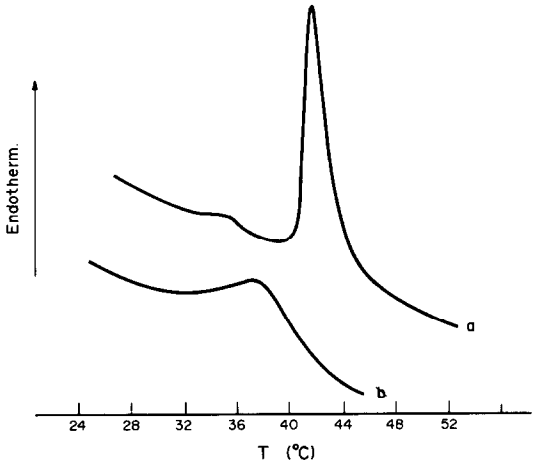

Fig. 1. DSC profile for pure DPPC MLV (a) and DPPC MLV containing 20 (mol.%) of amiodarone (X_I) (b). pH 7.4 (NaCl) = 0.15 M.

France) calorimeter, operating at a heating rate of 2°K/min. Multilamellar vesicles containing different molar fractions of amiodarone were prepared as described above at a lipid concentration of 55 μmol/ml. 100 μl aliquots of lipid suspension were placed in sealed stainless steel sample pans. A reference sample was similarly prepared using 100 μl buffer.

RESULTS AND DISCUSSION

DSC provide valuable information on the transition temperature and the enthalpy of melting characterizing lipid vesicles [16–18]. Figure 1(a) shows a classical pattern observed for DPPC multilamellar vesicles with a pretransition at 35.4° and a highly cooperative transition at 41.7°. For amiodarone containing liposomes (20% drug molar ratio) the pretransition vanishes and the peak associated to the main transition (Tr) is shifted to lower temperatures (Fig. 1b). This temperature shift is related to the amiodarone concentration (Fig. 2b). The observation that the temperature shift is accompanied by a decrease (Fig. 2a) of the enthalpy of melting (ΔH) suggests that amiodarone modifies the lipid conformation [19].

In order to analyze the influence of amiodarone ionization on the lipid transition temperature and the lipid enthalpy of melting, MLV were prepared at different pH (Fig. 2). The most striking modification of those two parameters was observed at the lower pH, i.e. for liposomes containing amiodarone in its ionized form. The procedure used to calculate the number of charged amiodarone moles ($n_{IH^{(+)}}$)

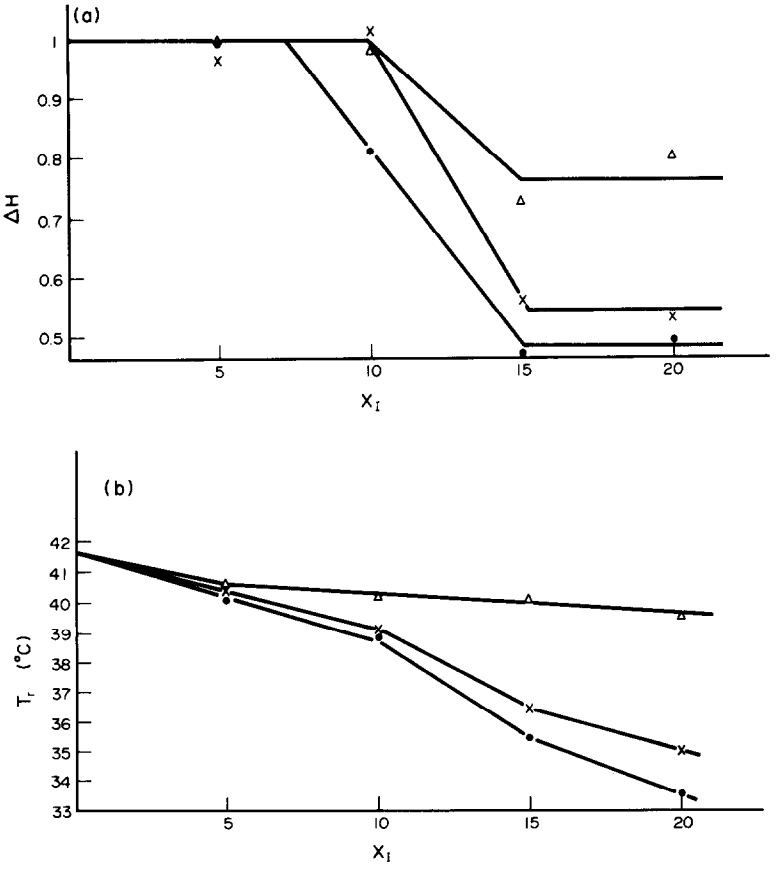

Fig. 2. Enthalpy of melting (ΔH) and transition temperature (Tr) as a function of the amiodarone molar fraction (X_I). The ΔH values are arbitrary values (ΔH = 1 for pure DPPC MLV). (NaCl) = 0.15 M; ● pH 4.3; × pH 7.4; △ pH 8.8.

inserted into the lipid vesicles requires the knowledge of the intrinsic ionization constant of amiodarone (pK_i) and the area (A) occupied per amiodarone molecule in the close packed state. These values have been obtained elsewhere [20].

The procedure can be described as follows. For amiodarone incorporated into liposomes, at different pH values, we consider the following lipid–water interfacial reaction [21]:

$$IH^{(+)} \rightleftharpoons I + H^{(+)}$$

where $IH^{(+)}$ and I correspond respectively to the acid and the basic form of amiodarone inserted into the vesicles. $H^{(+)}$ (or $H_3O^{(+)}$) can be exchanged with the aqueous subphase.

The intrinsic equilibrium constant of this interfacial reaction is given by

$$K_i = \frac{[I][H^{(+)}]_s}{[IH^{(+)}]} \quad (1)$$

$[H^{(+)}]_s$ is the $H^{(+)}$ concentration at the liposome interface. The total number of molecular species at the membrane interface can be defined as follows

$$\Sigma_i N_i = N_L + N_I + N_{IH^{(+)}} \quad (2)$$

where N_L, $N_{IH^{(+)}}$ and N_I are respectively the number of molecules of DPPC, of the acid and basic form of amiodarone.

The ionization degree of amiodarone is given by

$$\alpha = \frac{N_{IH^{(+)}}}{N_{IH^{(+)}} + N_I} \quad (3)$$

From combination of relations 1 and 3, it comes

$$pK_i = pH_s + \log \frac{\alpha}{1-\alpha} \quad (4)$$

where pH_s is the surface pH, related to the bulk pH_∞ through a Boltzmann distribution:

$$pH_s = pH_\infty + \frac{e\Psi}{2.3\,kT} \quad (5)$$

e is the electronic charge, k the Boltzmann constant, T the absolute temperature and Ψ the electrostatic potential at the interface.

The electrostatic potential (for amiodarone $\Psi > 0$) associated to the interface is given by the Gouy–Chapman theory of the electrical double layer [22]

$$\Psi = \frac{2kT}{Ze} \operatorname{sh}^{-1} \frac{e\sigma}{(8RT\varepsilon_r\varepsilon_0 C_i)^{1/2}} \quad (6)$$

R is the gaz constant, Z the valence of the ion, ε_r the relative permittivity of the solution, ε_0 the permittivity of the vacuum, C_i the ionic bulk concentration (mol/l) and σ the number of charged groups per surface unit. The $\Psi = f(\sigma)$ relationship (at given C_i value) is not significantly affected (this remark also concerns relation 1), by small variations of T. In our system, σ is given by the relation

$$\sigma = \frac{N_{IH^{(+)}}}{\Sigma_i A_i N_i} \quad (7)$$

where $\Sigma_i A_i N_i$ is the total surface area occupied per lipid and amiodarone molecules. From relation 2 and relation 7, comes

$$\sigma = \frac{N_{IH^{(+)}}}{N_L A_L + N_I A_I + N_{IH^{(+)}} A_{IH^{(+)}}} \quad (8)$$

where A_L, $A_{IH^{(+)}}$ and A_I are respectively the areas occupied per molecule of DPPC, acid form and basic form of amiodarone in the close packed state. Since the area occupied per molecule of amiodarone in a close packed state does not depend on the ionization degree [20]

$$A_I = A_{IH^{(+)}} \quad (9)$$

and

$$N_L A_I + N_{IH^{(+)}} A_{IH^{(+)}} = A_I N_I^T \quad (10)$$

with

$$N_I^T = N_I + N_{IH^{(+)}} \quad (11)$$

From relations (8) to (11)

$$\sigma = \frac{N_{IH^{(+)}}}{N_L A_L + N_I^T A_I} = \frac{N_{IH^{(+)}}}{\bar{A}(N_L + N_I^T)} \quad (12)$$

where

$$\bar{A} = X_L A_L + X_I A_I, \quad (13)$$

and X_L and X_I are respectively the molar fraction of DPPC and amiodarone in the liposome.

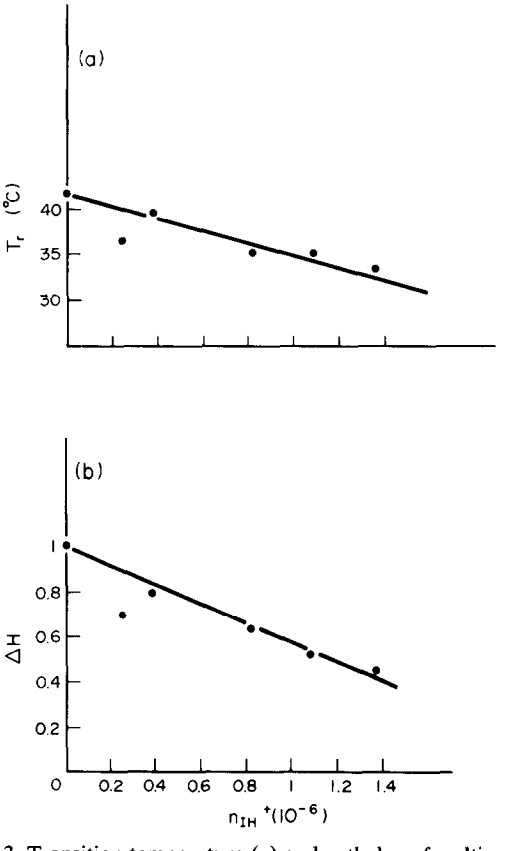

Fig. 3. Transition temperature (a) and enthalpy of melting (b) of DPPC MLV containing 20 mol.% of amiodarone X_I as of function of the number of charged amiodarone moles $n_{IH^{(+)}}$; $pK_i = 8.7$.

Table 1. Evolution of the transition temperature (Tr) and enthalpy of melting (ΔH) versus the ionization degree (α) and the number of charged amiodarone moles ($n_{IH^{(+)}}$) calculated for $pK_i = 8.7$ at different pH and for a molar fraction of 20 mol.% amiodarone X_I

Liposome	pH	α*	$n_{IH^{(+)}}$† (10^{-6})	Tr (Fig. 2b)	ΔH‡ (Fig. 2a)
DPPC	7.4	0	0	41.7	1
Amiodarone/DPPC	4.3	1	1.37	33.4	0.46
	5	1	1.37	33.6	0.45
	6	1	1.37	33.5	0.47
	7.4	0.8	1.09	35	0.53
	8.1	0.6	0.82	35	0.64
	8.8	0.28	0.38	39.5	0.8

* See the procedure of calculation described in the text.

† $n_{IH^{(+)}} = \alpha n_I^T$; n_I^T is the total number of moles of amiodarone incorporated in vesicles, for 20 mol.% amiodarone, $n_I^T = 1.37\ 10^{-6}$.

‡ The ΔH values are arbitrary considering the ΔH of pure DPPC vesicles as unity.

Table 2. Transition temperature versus the ionization degree α and the number of charged amiodarone moles ($n_{IH^{(+)}}$) calculated for $pK_i = 8.7$ at different pH and molar fractions of amiodarone (X_I)

		pH 7.4			pH 8.8			Ph 4.3		
X_I	n_I^T (10^{-6})	Tr	α	$n_{IH^{(+)}}$ (10^{-6})	Tr	α	$n_{IH^{(+)}}$ (10^{-6})	Tr	α	$n_{IH^{(+)}}$ (10^{-6})
0.05	0.29	40.4	0.92	0.27	40.5	0.38	0.11	40.1	1	0.29
0.10	0.61	39.1	0.88	0.54	40.2	0.34	0.21	38.8	1	0.61
0.15	0.96	36.4	0.83	0.79	40	0.31	0.3	35.4	1	0.96
0.20	1.37	35	0.8	1.09	39.6	0.28	0.38	33.5	1	1.37

From relations (3), (11) and (12) comes

$$\sigma = \frac{\alpha X_I}{A} \tag{14}$$

The intrinsic ionization constant of amiodarone ($pK_i = 8.7$) and the area occupied per molecule of amiodarone in the close packed state ($A_I = 44$ Å^2) are known [20]. Since the molecular area at the lipid–water interface of DPPC is equal to 60 Å^2 [23, 24] knowledge of pK_i, A_I and A_L values allows α and $n_{IH^{(+)}}$, to be determined using equations (4), (5), (6) and (14), for different molar fractions of amiodarone and at different pH values.

Figures 3(a) and (b) give respectively the Tr and the ΔH values as a function of $n_{IH^{(+)}}$, calculated for DPPC MLV containing 20% moles of amiodarone at different pH (Table 1). A linear relationship is obtained between ΔH or ΔTr and $n_{(IH)^+}$. No experiments were performed at pH values higher than 9, since preliminary free-flow electrophoresis measurements indicated that at these pH values DPPC can no more be considered as neutral (data not shown).

This modification of the DPPC properties above pH 9 is illustrated by the deviation from a linear relationship observed when Tr or pure DPPC MLV is plotted against the pH of the medium (Fig. 4). Since the extrapolated Tr and ΔH values at $n_{IH^{(+)}} = 0$ are those of vesicles made of pure DPPC (Fig. 3) and since ionized and unionized amiodarone molecules were shown to be totally incorporated into MLV [25], one can assume that the ionized form

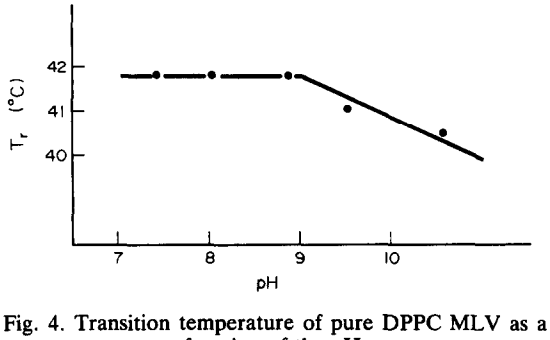

Fig. 4. Transition temperature of pure DPPC MLV as a function of the pH.

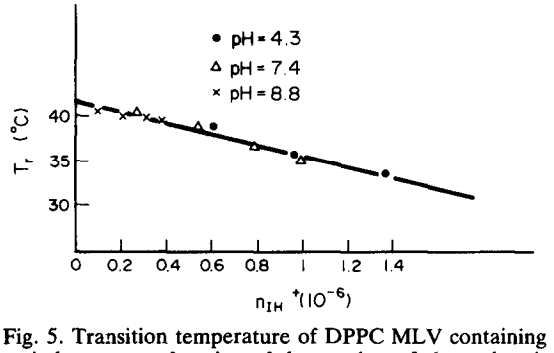

Fig. 5. Transition temperature of DPPC MLV containing amiodarone as a function of the number of charged amiodarone moles; $pK_i = 8.7$.

Table 3. Transition temperature versus the ionization degree and the number of charged amiodarone moles ($n_{IH^{(+)}}$) calculated for $pK_i = 7.7$ at different pH and molar fractions of amiodarone (X_I)

		pH 7.4			pH 8.8			pH 4.3		
X_I	n_I^T (10^{-6})	α	$n_{IH^{(+)}}$ (10^{-6})	Tr	α	$n_{IH^{(+)}}$ (10^{-6})	Tr	α	$n_{IH^{(+)}}$ (10^{-6})	Tr
0.05	0.29	0.6	0.174	40.4	0.071	0.020	40.5	1	0.29	40.1
0.10	0.61	0.53	0.323	39.1	0.068	0.041	40.2	1	0.61	38.8
0.15	0.96	0.48	0.46	36.4	0.066	0.063	40	1	0.96	35.4
0.20	1.37	0.43	0.509	35	0.06	0.082	39.6	1	1.37	33.5

of amiodarone has the capability of modifying the transition temperature and the enthalpy of melting. Interestingly, a linear relationship between Tr and $n_{IH^{(+)}}$ is obtained (Fig. 5 and Table 2) whatever the pH value of the buffer for the pK_i value ($pK_i = 8.7$) of amiodarone that has been determined from the surface potential approach [20]. For a given amiodarone molar function and a given pH, $n_{IH^{(+)}}$ depends only on the pK_i value. On the contrary, for another value given to pK_i ($pK_i = 7.7$) a distinct linear relationship between Tr and $n_{IH^{(+)}}$ is obtained at each pH (Fig. 6 and Table 3) which indicates that this pK_i chosen value is incorrect. Only one pK_i value leads to a unique relationship between Tr and n_{IH^+} whatever the pH. To apply this new procedure of evaluation of pK_i to water insoluble molecules, two conditions must be fulfilled:

the drug must be totally inserted into the lipid membrane;

one of its two forms only (protonated or unprotonated) should be capable of modifying the lipid organization (Tr, ΔH).

The accuracy of this determination is within 0.5 pK_i unit.

Several explanations can be proposed in order to explain the capability of the ionized form of amiodarone to destablize the lipid organization. First, it can be assumed that the ionization of the amiodarone molecule mediates a structural change that perturbs the conformation of its lipidic environment. It is known indeed that even a minor modification can drastically enhance the drug capacity to destabilize the membrane structure.That point has been illustrated for ketoconazole which does not affect significantly the lipid organization of DL-α-dipalmitoylphosphatidylcholine (DPPC) [26]. Deacylation of this molecule modifies its orientation into the lipid bilayer and increases the destabilizing capability of the drug as demonstrated by DSC measurements. A shift of 10° of the mean DPPC transitions was observed, whereas no significant shift was obtained with ketoconazole inserted at equal concentration into the DPPC bilayer. Since the area occupied per amiodarone molecule in the neutral and charged state are identical when assembled in a close-packed monolayer spread at the air–water interface [20], the possibility of an amiodarone structural change seems unlikely. One can not exclude, however, that even if the drug structure does not change, its penetration into the lipid layer could be mediated by its ionization state. IR spectroscopy, (Attenuated Total Reflection Technique) and conformational analysis are envisaged in our laboratory as possible tools to solve this problem.

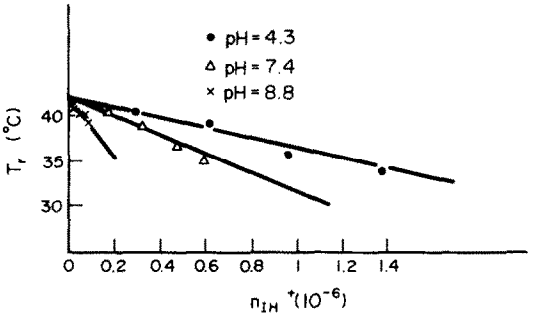

Fig. 6. Transition temperature of DPPC MLV containing amiodarone as a function of the the number of charged amiodarone moles. $pK_I = 7.7$.

Another assumption is that neutral amiodarone molecules are organized in clusters which minimize the interaction with lipids and consequently the destabilization. Drug ionization responsible for the repulsion between drug molecules, would favour their random distribution into the lipid matrix. The possibility given to each amiodarone molecule to interact with the surrounding lipids would increase the lipid disorganization. Such a mechanism has been proposed to explain the increasing effect as a function of the decrease in pH of a constant amount of amiodarone on the temperature phase transition of DPPC MLV visualized by fluorescence depolarization [8]. The present finding that the amiodarone ionization state mediates its interaction with the lipids might contribute to elucidate the amiodarone mode of action since electrostatic interactions have been shown to modulate antiarrhythmic drug interaction with the lipid [4, 5] as well as with the protein components [3, 4] of the membrane.

Acknowledgements—This work was partly supported by a grant from Institut pour l'Encouragement à la Recherche Scientifique dans l'Industrie et l'Agriculture.

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