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AMIODARONE INDUCED MODIFICATIONS OF THE PHOSPHOLIPID PHYSICAL STATE

A FLUORESCENCE POLARIZATION STUDY

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Abstract—The effects of an antiarrhythmic and antianginal drug, amiodarone, on the physical state of membrane phospholipids was investigated by means of fluorescence polarization using the apolar probe 1,6 diphenyl-1,3,5-hexatriene incorporated in the hydrocarbon core. Multilamellar vesicles were prepared from neutral phospholipids (egg phosphatidylcholine, synthetic saturated phosphatidylcholine) alone or mixed with cholesterol or various lipids representative of the main lipid classes. 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. In the gel state, the lipid mobility depends on drug concentration, degree of ionization and the length of the lipid acyl chains. In the liquid-crystalline state, the decreased lipid mobility which is concentration-dependent is essentially due to hydrophobic interactions. Amiodarone increases the lipid order parameter to the same extent as cholesterol. The data suggested that amiodarone is a rigid molecule deeply buried in the hydrocarbon core of the lipid and that amiodarone–lipid interactions are mainly hydrophobic.

Amiodarone is used as an antianginal and antiarrhythmic drug; its efficacy in the treatment of a wide spectrum of arrhythmias is well documented [1]. However, its mode of action is poorly understood. Nevertheless, several experimental observations such as micelle formation [2], a high degree (≥95%) of fixation to serum proteins [3, 4], rapid penetration in tissues [5, 6] suggest low water-solubility and significant hydrophobic behaviour. These aspects were documented recently by the determination of (1) the amiodarone–lipid partition coefficient, the log value of the neutral form of the drug being 5.95 [7] and of (2) the effects of the drug on lipid dynamics and enzyme activation in rat-brain synaptic membranes [8]. In the latter study, the inhibitory effect exerted by amiodarone on the Na^+/K^+ ATPase has been attributed, at least in part, to the effect of the drug on the lipid dynamics [8]. This effect is unusual in that the drug decreased lipid fluidity. This prompted us to characterize further the lipid–amiodarone interactions. In the present communication, the drug induced fluidity modifications were studied in multilamellar vesicles (MLV)‡ by means of a fluorescence polarization technique using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe incorporated in the hydrocarbon core of the bilayer [9]. MLV were made of phosphatidylcholine alone or mixed with cholesterol or phospholipid of the main classes found in natural membranes. The drug effects were studied as a function of drug concentration, temperature, pH and vesicle composition.

MATERIALS AND METHODS

Materials

Amiodarone (Cordarone®, Labaz-Sanofi, 2-butyl-3-[4-(2-diethylaminoethoxy)-3′,5′-diiodo-benzoyl]-benzofuran, M.W.: 660) was obtained from the Chemical Department. Its chemical structure is depicted in Fig. 1. Lipids (dimyristoyl-DL-α-phosphatidylcholine, DMPC, dipalmitoyl-DL-α-phosphatidylcholine, DPPC, distearoyl-DL-α-phosphatidylcholine, DSPC, egg phosphatidylcholine from egg yolk (type VII–E), Egg PC, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidic acid, cardiolipin, phosphatidylethanolamine and cholesterol) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma Chemical Co (St Louis, MO). Purity of the lipids was analyzed by thin-layer chromatography on silica gel G plates using chloroform, methanol, ammoniac as solvent in the volume ratio 13:5:1 and staining

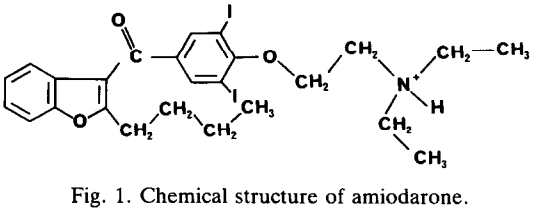

Fig. 1. Chemical structure of amiodarone.

‡ Abbreviations used: Am, amiodarone; Ch, cholesterol; DMPC, dimyristoyl-DL-α-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-DL-α-phosphatidylcholine; DSPC, distearoyl-DL-α-phosphatidylcholine; MLV, multilamellar vesicle; Egg PC, phosphatidylcholine from egg yolk; T_m, main phase transition temperature.

with iodine. Lipids were used without further purification. Distilled and deionized water was used throughout the experiments. All other reagents were of the highest purity commercially available.

Methods

Preparation of samples. Multilamellar vesicles (MLV) were prepared from individual phospholipids such as DMPC, DPPC, DSPC of Egg PC or from mixtures containing phosphatidylcholine plus the other classes of lipids at various ratios. A chloroform solution containing the required lipid composition, DPH in a molar 1:500 ratio and various amounts of amiodarone was evaporated to dryness under a stream of N_2. Trace amounts of solvent were removed under reduced pressure. The thin film which formed on the walls of the flask was dispersed in a Tris–HCl buffer (20 mM, pH: 7.4, NaCl 0.134 M), unless otherwise stated, by mechanical agitation (Vortex 5 min) at room temperature or at a temperature above the main phase transition temperature (T_m) of the phospholipid [10]. MLV were washed by successive centrifugations (10,000 *g*, 10 min) and resuspensions in the same buffer. In control experiments, the recovery of phospholipids and amiodarone was checked by determining inorganic phosphate [11] and ^{14}C amiodarone [7]. Phospholipids and amiodarone recovery were both >95%. The final lipid concentration was adjusted to around 0.2 mg/ml.

Fluorescence polarization measurements. The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for monitoring the degree of fluidity of the hydrocarbon core of the lipid bilayer. Excited-state lifetimes (τ) of DPH were determined with the SLM 4800 Spectrofluorimeter. Steady state fluorescence polarization was measured using either an Elscint Microviscometer, Model MV 1 (Elscint Ltd., Haifa, Israel) or a SLM 4800 Spectrofluorimeter (SLM Instruments, Champaign, IL). Both apparatus were working in the T format in which the polarized light emitted is detected in two independent cross-polarized channels after passing through cut-off filters ($\lambda < 418$ nm) or a monochromator ($\lambda_{em} = 450$ nm). The steady-state fluorescence polarization (P) is defined as follows:

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \quad (1)$$

where $I_\parallel$ and $I_\perp$ are the intensities of the light emitted with its polarization plane parallel ($\parallel$) and perpendicular ($\perp$) to that of the exciting beam. Corrections for light scattering were made by using reference samples without DPH [9]. Temperature of the samples kept within 0.2°. The results are the mean of two determinations performed on two to four individual multilamellar vesicles preparations. Standard deviation, omitted for clarity, is 1% or better.

RESULTS

According to the Perrin equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{\tau \cdot RT}{V\eta}\right) \quad (2)$$

where P is the measured fluorescence depolarization, P_0 is the limiting value of P, τ is the fluorescence lifetime, V is the effective molar volume of the probe, η is the viscosity of the medium, the movements of the probe within the medium depend on both τ and η.

It is generally assumed that τ is constant at one temperature and thus P is directly related to η. However, the presence of a third compound can modify τ if this compound acts as a quencher. This direct measurement of τ in the absence of a quencher enables this possibility to be verified. Although there is a trend towards a decrease in τ, no significant decrease was found up to 25% of amiodarone incorporated in a phospholipid matrix. Consequently, the observed variations in P values do represent variations in DPH mobility and thus in lipid dynamics.

Figure 2 shows a typical fluorescence depolarization versus temperature scan of a MLV suspension prepared from DPPC without and with increasing amounts of amiodarone. The DPPC scan is characterized by two transitions determined graphically at 42° and 33°. The sharp main transition in temperature (T_m) corresponds to a phase transition, i.e. from the gel to the liquid-crystalline state. Below T_m, the fatty acid chains are packed in a highly ordered array; this corresponds to a restricted motion of DPH and to high P values. Above T_m, the chains are disordered and possess more motional freedom. This leads to increased mobility of DPH and to low P values. Incorporation of amiodarone is responsible for the disappearance of the lower transition temperature (for amount superior to 2%), and to dose-dependent downward shift of the main transition temperature (Fig. 2 insert), a decrease of P when DPPC is in the gel state and an increase of P when DPPC is in the liquid-crystalline state. The downward shift of the main transition temperature, usually taken as the main criterion to characterize the disordering effect of a drug [12, 13], reaches a plateau for approximately 15% of amiodarone, i.e. one drug molecule for six phospholipids (Fig. 2 insert). It can be hypothesized that, in these experimental conditions, this corresponds to the maximal effect on lipid dynamics, the latter being packed in a highly ordered hexagonal assay.

The differential effect of amiodarone on lipid dynamics has been further investigated using a series of phosphatidylcholines (DMPC, DPPC, DSPC, Egg PC) and measuring the effect of increasing amounts of the drug at various temperatures (Fig. 3). When the lipid is in the gel state (DMPC, DPPC and DSPC at 15°, DPPC and DSPC at 25°, DSPC at 45°), amiodarone induces a dose-dependent decrease in P, i.e. an increase in lipid mobility. These results confirm and extend the data shown in Fig. 1 for DPPC below T_m. They are along the same line as the depression of the phase-transition temperature induced by the compound (Fig. 2 insert). Thus the effects of amiodarone on both the phase-transition temperature and lipid mobility in the gel state suggest that the drug upsets the highly ordered lipid organization and increases fluidity of the lipid matrix.

On the other hand, when the phospholipid is in the liquid-crystalline phase (Egg PC at all temperatures studied, DMPC at 25°, DMPC and DPPC at 45°,

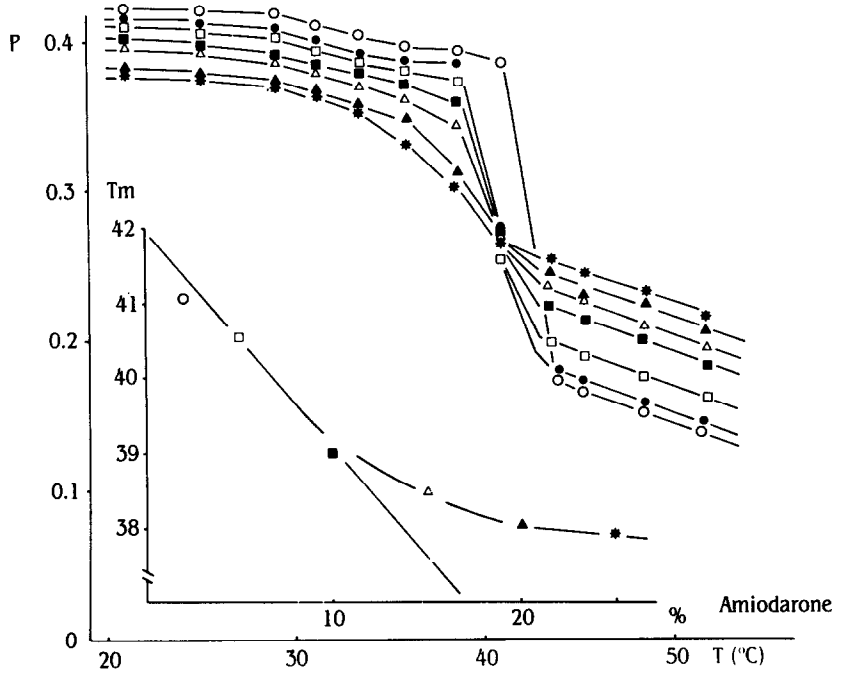

Fig. 2. Fluorescence depolarization scan of DPH in DPPC MLV in the absence (○) and in the presence of increasing amounts of amiodarone (●, 2%; □, 5%; ■, 10%; △, 15%; ▲, 20%; ×, 25%). The quantity of amiodarone is expressed in per cent of the quantity of DPPC. Insert: change of the main transition mid-point temperature (T_m) as a function of amiodarone concentration.

DMPC, DPPC and DSPC at 60° (not shown)), amiodarone induces a dose-dependent decrease in P, i.e. reduces lipid mobility. These observations confirm and extend the data shown in Fig. 2 for DPPC above T_m. Thus, at the opposite to the gel state, amiodarone is able, in the liquid-crystal phase, to restrict the motional freedom of the hydrocarbon chains. It must be emphasized that the downward shift in the main phase-transition temperature (Fig. 2 insert) and the decrease in fluidity (Fig. 3 upper

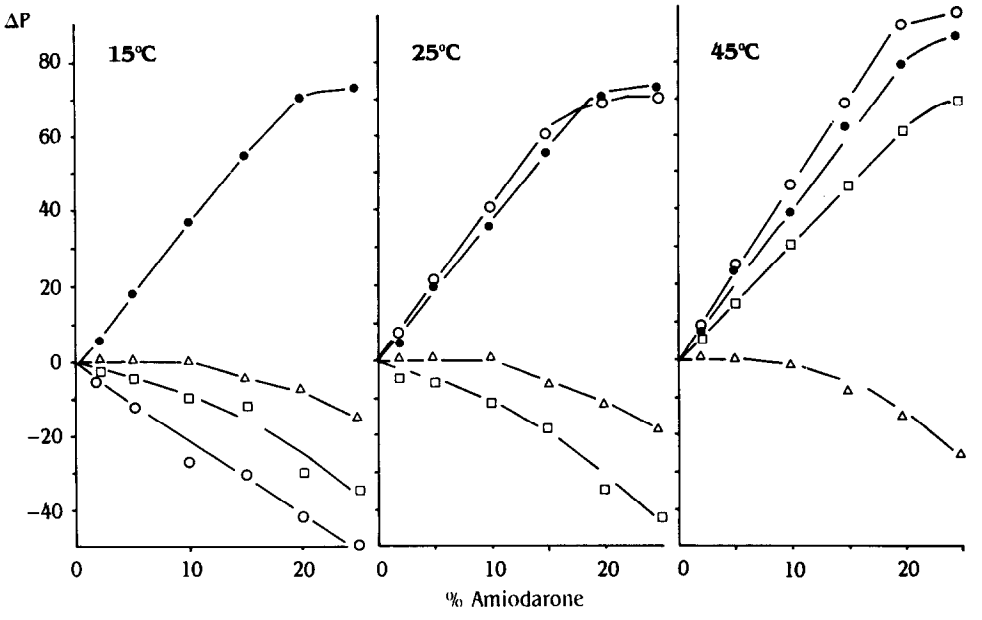

Fig. 3. Variation (ΔP) of fluorescence depolarization of DPH as a function of amiodarone concentration. ΔP is defined as the difference of fluorescence depolarization in the presence (P_{am}) and in the absence (P_0) of amiodarone, $\Delta P = P_{am} - P_0$. The effect of amiodarone on the following phospholipids: Egg PC (●), DMPC (○), DPPC (□) and DSPC (△), was studied at 15° (left panel), 25° (middle panel) and 45° (right panel).

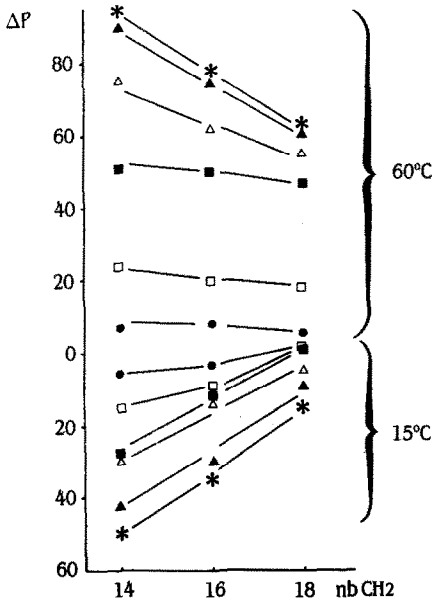

Fig. 4. Influence of the length of the lipid acyl chains (DMPC, 14 C; DPPC, 16 C; DSPC, 18 C) on the effect of amiodarone in both gel (15°) and liquid crystalline (60°) states. Amiodarone concentration: ●, 2%; □, 5%; ■, 10%; △, 15%; ▲, 20%; *, 25%.

panels) are linear with the amiodarone concentration up to ≅15% and then tend towards a plateau. Such a plateau is not observed when the phospholipid is in the gel state. Both fluidification and rigidification effects of amiodarone are further quantified in Fig. 4 when the effects of increasing amounts of the compound are plotted as a function of lipid chain-length at two temperatures. These temperatures correspond to the presence of either the gel state (15°) or the liquid-crystalline state (60°) for each lipid. Two observations can be made: (a) the effect of amiodarone depends, in both states, on the hydrocarbon chain-length: the longer the chain, the smaller the effect; (b) although, at a given amiodarone proportion, the variation in fluidity is roughly equivalent in both states, the absolute value of the decrease in mobility is greater than the increase, the latter being half the value of the former.

Bulk pH has a pronounced influence on the effects of amiodarone on the DPPC MLV phase transition and on lipid fluidity in the gel state (Fig. 5), the decrease in the main transition temperature and the increase in fluidity augmenting with the degree of ionization (the pK_i of amiodarone is 8.7 [14]). These effects cannot be attributed to variations in amiodarone concentration in the bilayer since the partition coefficient of the compound is insensitive to the bulk pH (P. Chatelain, unpublished results). When the lipid is in the liquid-crystal state, the effect of the compound does not vary whatever the degree of ionization. In the range of pH values studied, few effects of the pH on the fluorescence depolarization scans of DPPC were observed.

Various phospholipids from natural sources (alone or mixed) in amounts corresponding to that found in purified heart sarcolemmal membrane [15] were incorporated in Egg PC MLV. With the exception of cholesterol, these additions have little effect on the fluidity of Egg PC in the absence and in the presence of a fixed amount of amiodarone (Fig. 6).

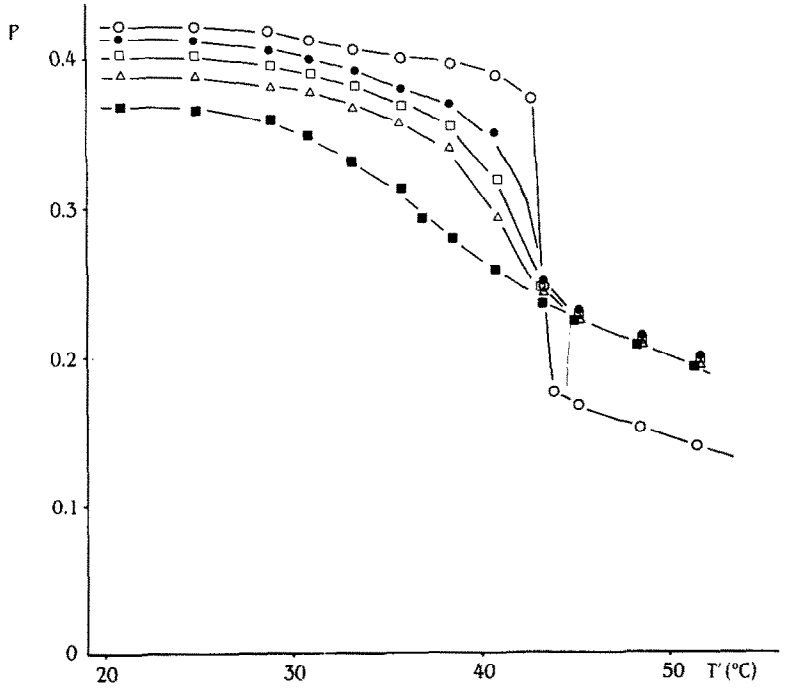

Fig. 5. Influence of the bulk pH on the effects of amiodarone on the fluorescence depolarization scan of DPH in DPPC MLV: ○, control scan in the absence of amiodarone; ●, □, △, ■, scans at the following pH 8.8, 8.1, 7.4, 4.4 in the presence of 15% amiodarone.

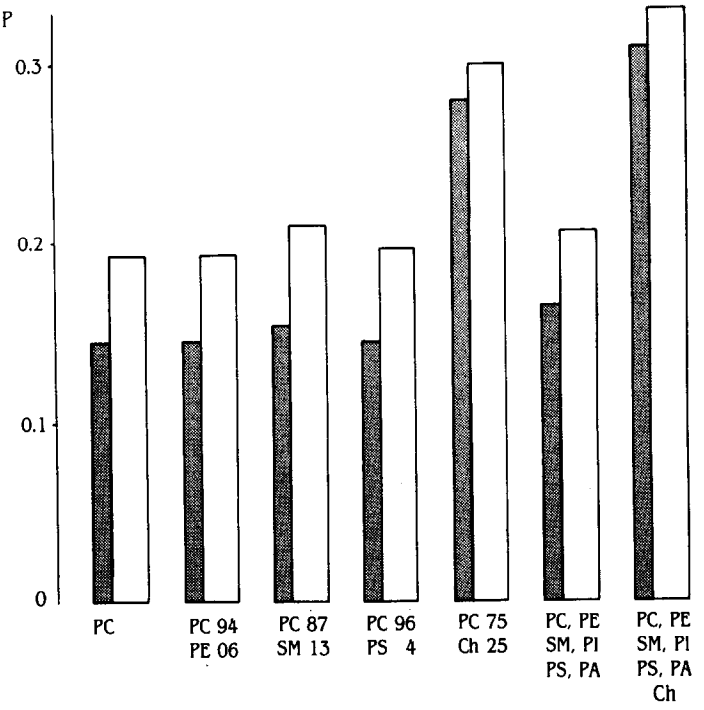

Fig. 6. Influence of lipid composition on the effect of amiodarone on the degree of fluorescence depolarization. Amiodarone (open column) is incorporated at a fixed amount (15%). PC, PE, SM, PS, PI, Ch represent respectively the Egg phosphatidylcholine, phosphatidylethanolamine, sphingomyeline, phosphatidylserine, phosphatidylinositol and cholesterol at the indicated amounts. In the last two mixtures, the lipids were mixed in amounts found in purified heart sarcolemma [15].

The same result was obtained when 6% phosphatidylinositol, 3% phosphatidic acid and 3% cardiolipin were incorporated in Egg PC (not shown). Cholesterol (25%) induced a marked increase in viscosity in Egg PC MLV which is even greater on the complete lipid mixture. Amiodarone still decreases the viscosity but to a lesser extent than in the absence of cholesterol.

Based on the observations that the depolarizing rotations of DPH are anisotropic in a lipid bilayer, Heyn [16], Jähnig [17] and Van Blitterswijk *et al.* [18] improved the usefulness of P measurement by relating it to the lipid parameter order S_V, where V indicates the mean position of DPH along the fatty acid chains. They established that the steady state fluorescence anisotropy r_s

$$\left(r_s = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} = \frac{2P}{3 - P}\right)$$

is divided into a first decaying dynamic component r_f and an infinitely slow decaying component r_∞, the latter yielding an average lipid order parameter s_V:

$$r_s = r_f + r_\infty \quad (3)$$

$$r_\infty = (1 + \gamma)r_s - \gamma r_0 \quad (4)$$

$$S_V = \sqrt{\left(\frac{r_\infty}{r_0}\right)} \quad (5)$$

where r_0 is the limiting fluorescence anisotropy (0.39 in ref. 17) and γ the rotational correlation time of the probe (ϕ) divided by its fluorescence lifetime (τ). From these observations, Van Blitterswijk *et al.* [18] presented the following empirical relation between r_s and r_∞ which is used below:

$$r_\infty = \frac{4}{3}r_s - 0.10$$

The effect of amiodarone and cholesterol (alone or mixed) on the order parameter S_V of phosphatidylcholine MLV in the liquid-crystalline state (Egg PC 25°, DPPC 50°) is compared in Fig. 7. In Egg PC MLV, control values in the absence and presence of cholesterol are in excellent agreement with published data [16] (for a review see ref. 17). Amiodarone alone or mixed with cholesterol at various concentrations has the same effect on the structural order parameter. Other Egg PC/cholesterol/amiodarone mixtures have been tested. As they gave similar results, they have been omitted from Fig. 7 for clarity. The same observation is performed with DPPC. Interestingly, S_V tends towards a limiting value $0.8 < S_v < 0.9$ at 25° which is in agreement with the previous data obtained on natural membranes [18].

DISCUSSION

Steady state fluorescence polarization data demonstrated that amiodarone has a fluidizing effect below the phase transition temperature (gel state) of phospholipids and a rigidizing (ordering) effect above this temperature (Figs 2, 5–7). These effects depend on the drug concentration and ionization

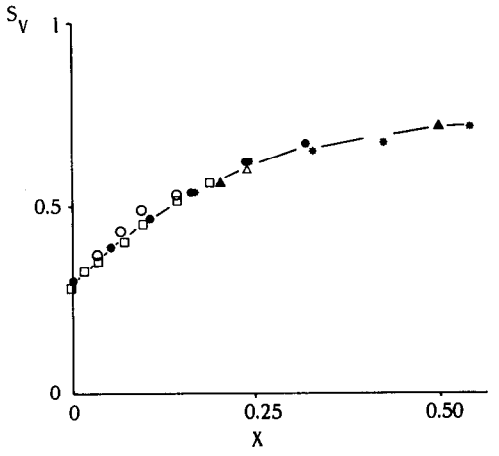

Fig. 7. Comparison of the influence of amiodarone and cholesterol on the structural order parameter S_V for the phosphatidylcholines MLV: ○, Egg PC + amiodarone; ●, Egg PC + cholesterol; ×, Egg PC/Am 10% + cholesterol; □, DPPC + amiodarone at 50°; ▲, Egg PC + cholesterol calculated from Shinitzky and Inbar [19]. Measurements on Egg PC MLV were performed at 25°. × is the molar fraction of amiodarone or cholesterol.

(Figs 2–5) and the length of the lipid acyl chains (Figs 3, 4). Such a spectrum of effects on lipid dynamics is observed with long chain alcohols [20, 21] and carboxylic acid [22] or cholesterol [19, 22]. These compounds are buried deeply in the hydrocarbon core which promotes Van der Waals' forces with the acyl chain. Amiodarone (Fig. 1) can be viewed as a bulky molecule composed of a large hydrophobic nucleus and a small hydrophilic tail.

This present data suggest that amiodarone penetrates deeply into the lipid matrix. Due to the amphiphilic nature of the molecule, the hydrophobic nucleus is likely to be located along the lipid acyl chains and the amino residue at the lipid–water interface. The idea that the hydrophobic forces in the amiodarone–lipid interaction predominate is reinforced by several observations. Firstly, the addition of acid phospholipids in the Egg PC matrix does not modify the effects of amiodarone on lipid fluidity (Fig. 6) as shown for other amphiphilic compounds [10, 23]. Secondly, the variation of bulk pH does not modify the effects of amiodarone in the liquid-crystalline state. A similar observation was made by comparing the effects of a series of analogues of cholesterol on microviscosity parameters. Although the analogues were dipolar, positively or negatively charged, they induced an increase in microviscosity parameter similar to that of cholesterol in fluid lipid matrices [24–26] which implies that the nature of the hydrophilic region of the lipid bilayer is of secondary importance in the submacroscopic view of the lipid fluidity determined by fluorescence depolarization [26]. The situation is different in the gel state where the effect of amiodarone increases as the pH decreases (Fig. 5). It may be hypothesized that amiodarone and DPPC in the gel state mix unideally: amiodarone should thus separate out from the DPPC phase. This is, however, counteracted by the repulsion between the positive amino-groups of amiodarone, the latter effect being linked to the degree of ionization of the drug. Thirdly, in the range of concentration investigated, amiodarone has a pronounced effect on the structural order parameter (S_V). In the liquid-crystalline state, amiodarone produces an increase in S_V, i.e. an ordering of the lipid acyl chains. Furthermore, this effect is identical to that of cholesterol in the same experimental conditions. Thus amiodarone seems to be able to reduce the molecular packing (or the motional freedom) of the lipid acyl chains to the same extent as cholesterol. Since cholesterol is a rigid molecule located along the lipid acyl chains with its long axis perpendicular to the bilayer plane [27], comparable characteristics of rigidity in the molecule and position in the lipid matrix can be proposed for amiodarone. Studies are in progress to verify these conclusions.

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REFERENCES

1. B. N. Singh, K. Nademanee, N. Ikeda and R. Kannan, in *New Aspects in the Treatment of Tachyarrythmias—Role of Amiodarone* (Eds. G. Breithardt and F Loogen), pp. 46–56 Urban and Schwarzenberg, München (1983).
2. R. J. Warren, R. J. Stedman, E. G. Shami, E. S. Rattie and L. J. Ravin, *J. Pharm. Sci.* **59**, 1357 (1970).
3. R. Charlier, G. Deltour, A. Baudine and F. Chaillet, *Arzneim.-Forsch.* **18**, 1408 (1968).
4. M. P. A. Lalloz, P. G. H. Bylfield and R. L. Himsworth, *J. Pharm. Pharmac.* **36**, 366 (1984).
5. E. Riva, M. Gerna, R. Neyroz, R. Urso, I. Bartosek and A. Guaitani, *J. Cardiovasc. Pharm.* **4**, 270 (1982).
6. M. M. G. Debbas, C. Du Cailor, A. Sassine, J. Derancourt, J. Demaille and P. Puech, *Eur. J. clin. Invest.* **13**, 123 (1983).
7. P. Chatelain and R. Laruel, *J. Pharm. Sci.* **74**, 783 (1985).
8. P. Chatelain, R. Laruel and M. Gillard, *Biochem. biophys. Res. Commun.* **129**, 148 (1985).
9. M. Shinitzky, A. C. Diamond, C. Gitler and G. Weber, *Biochemistry* **15**, 2106 (1971).
10. P. Chatelain and J. M. Ruysschaert, *Biochem. Pharmac.* **30**, 313 (1981).
11. K. Itaya and M. Ui, *Clin. chim. Acta* **14**, 361 (1966).
12. M. K. Jain and M. N. Wu, *J. memb. Biol.* **34**, 157 (1977).
13. D. B. Goldstein, *Ann. Rev. Pharmac. Toxic.* **24**, 43 (1984).
14. J. Ferreira, R. Brasseur, P. Chatelain and J. M. Ruysschaert, submitted to *J. Pharm. Pharmac.*
15. W. B. Weglicki, K. Owens, F. Kennett, A. Kessner, L. Harris and R. M. Wise, *J. biol. Chem.* **255**, 3605 (1980).
16. M. P. Heyn, *FEBS Lett.* **108**, 359 (1979).
17. F. Jahnig, *Proc. natn. Acad. Sci., USA*, **76**, 6361 (1979).
18. W. J. Van Blitterswijk, R. P. Hoeven and B. W. Van der Meer, *Biochim. biophys. Acta* **644**, 323 (1981).
19. M. Shinitzky and M. Inbar, *Biochim. biophys. Acta* **433**, 133 (1976).
20. M. J. Pringle and K. W. Miller, *Biochemistry* **18**, 3314 (1979).

21. A. W. Eliasy, D. Chapman and D. F. Ewing, *Biochim. biophys. Acta* **448**, 220 (1976).
22. S. Kawato, K. Kinosita and A. Ikegami, *Biochemistry* **17**, 5026 (1978).
23. A. G. Lee, *Molec. Pharmac.* **13**, 474 (1977).
24. M. Lyte and M. Shinitzky, *Chem. Phys. Lipids* **24**, 45 (1979).
25. I. Yuli, W. Wilbrandt and M. Shinitzky, *Biochemistry* **20**, 4250 (1981).
26. M. Shinitzky and I. Yuli, *Chem. Phys. Lipids* **30**, 261 (1982).
27. D. M. Engelman and J. E. Rothman, *J. biol. Chem.* **247**, 3694 (1972).