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Neutrality of Amiodarone on the Initiation and Propagation of Membrane Lipid Peroxidation

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Amiodarone is an iodinated benzofuran derivative largely used as an antiarrhythmic. Owing to the sensitivity of heart tissue to radicals, amiodarone was assayed for putative effects on lipid peroxidation studied in liposomes of soybean phosphatidylcholine and of bovine heart mitochondrial lipids used as model systems. Lipid peroxidations were initiated with Fe^{2+}/ascorbic acid, and with peroxyl radicals generated from the azocompounds, AAPH and AMVN. These assays were carried out by following the quenching of the fluorescent probe *cis*-parinaric acid and by monitoring oxygen consumption. It has been ascertained that amiodarone does not protect or potentiate significantly the lipid peroxidation in both lipidic systems. To fully ascertain the neutral behaviour of amiodarone in the lipid peroxidation process, the degradation of phospholipid acyl chains has been checked by GLC. These data confirm that amiodarone does not protect or potentiate lipid peroxidation to a significant extent. It is concluded that the limited effects of amiodarone might be related only indirectly with the lipid peroxidation. It is possible that the drug causes limited conformational and biophysical alterations in membrane phospholipid bilayers that can affect the process of peroxidation. Therefore, it is concluded that the therapeutic effects and benefits as a heart antiarrhythmic agent are independent of lipid peroxidation processes. Furthermore, the interaction of the drug with lipid bilayers does not induce significant conformational perturbations that could significantly favour or depress the peroxidation process.

KEY WORDS — amiodarone; liposomes; lipid peroxidation; *cis*-parinaric acid

ABBREVIATIONS — AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AMD, amiodarone; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); GLC, gas–liquid chromatography; HEPES, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; MDA, malondialdehyde; PnA, *cis*-parinaric acid; PUFA, polyunsaturated fatty acids; soybean PC, soybean phosphatidylcholine; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TRIS, tris(hydroxymethyl)aminoethane.

INTRODUCTION

Amiodarone [2-butyl-3-(3′,5′diiodo-4′α-diethylamino-ethoxy-benzoyl)-benzofuran] (Figure 1) is a potent antianginal and antiarrhythmic drug, that extends the duration of the action potential, thus opposing cardiac hyperexcitability.[1,2] Plasma amiodarone concentrations of approximately 1–2·5 μg ml^{-1} are usually necessary for suppression of arrhythmias.[3,4] Therapeutic effects are accompanied by a variety of side-effects[3] on the lungs, liver, nervous system, thyroid gland, cornea and skin.[3,5] A common feature of all these side-effects is related to the accumulation of the drug in a variety of tissues.[6] Long-term administration disturbs phospholipid metabolism, leading to cellular phospholipidosis secondary to phospholipase inhibition,[7] formation of free radicals and altered cellular function or immunological injury.[8] Amiodarone belongs to a class of cationic amphiphilic drugs with a large lipophilic moiety allowing strong interaction with membranes.[9]

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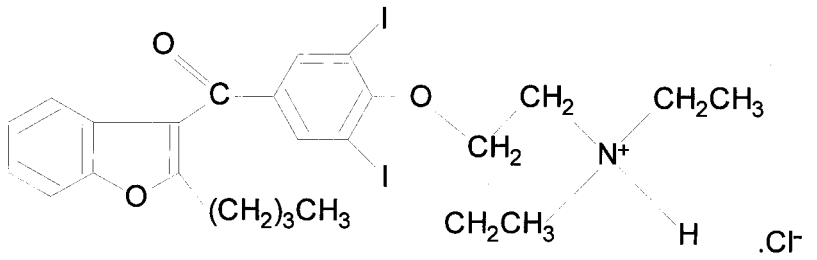

Figure 1. Amiodarone [2-butyl-3-(3′,5′diiodo-4′α-diethylamino-ethoxy-benzoyl)-benzofuran].

Peroxidation of cellular lipids is of significant interest owing to the evidence that peroxyl radicals and products of lipid peroxidation may be involved in the toxicity of compounds acting as prooxidants and antioxidants related to a variety of pathological conditions.[10] In this context, it has been reported by Rekka *et al.*[11] that amiodarone might inhibit non-enzymic lipid peroxidation induced by Fe^{2+}/ascorbic acid, on the basis of thiobarbituric acid-reactive material measured in inactivated rat hepatic microsomal fractions. Recently, Ribeiro *et al.*[12] reported that amiodarone (16·6 μM) inhibits, by about 70 per cent, the lipid peroxidation in rat liver mitochondria also assayed by the production of thiobarbituric acid-reactive substances. They also reported that amiodarone has no effect on the activity of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase, suggesting that amiodarone does not promote increased formation of superoxide anion dependent on mitochondrial redox activities. These findings prompted us to study the putative interaction of amiodarone on the mechanism of membrane lipid peroxidation, using sensitive and specific methodologies rather than the TBARS technique which is prone to several artifacts. Very often, most of the malondialdehyde (MDA) measured may be formed not during the peroxidation process, but during the breakdown of lipid peroxides in the acid-heating step, especially when metal catalysts are present.[13]

Therefore, the aim of our work was to evaluate precisely the interaction of amiodarone with lipid peroxidation processes monitored by *cis*-parinaric acid (PnA) fluorescence quenching, oxygen consumption and by degradation of phospholipid acyl chains evaluated by gas–liquid chromatography. The studies were carried out in model systems of soybean phosphatidylcholine liposomes and liposomes of lipids extracted from bovine heart mitochondria.

MATERIALS AND METHODS

Materials

cis-Parinaric acid was purchased from Molecular Probes (Junction City, OR, U.S.A.). Azocompounds AAPH and AMVN were purchased from Polysciences Inc. (Warrington, PA, U.S.A.). Amiodarone and L-α-phosphatidylcholine from soybean (99 per cent) were obtained from Sigma Chemical Co. (U.S.A.). All the other chemicals were obtained from Sigma Chemical Co. Solutions were prepared in deionized ultrapure water (Milli-Q) to minimize metal contamination.

Preparations

Amiodarone was dissolved in N,N-dimethylformamide, due to its low solubility in water. Control experiments in the presence of N,N-dimethylformamide were performed and no effect of the solvent was detected.

Beef heart mitochondria were prepared according to Low and Vallin.[14] Protein was measured according to Lowry *et al.*[15] against standards of serum albumin.

Lipids were extracted as described by Madeira and Antunes-Madeira.[16] The phospholipid concentration was determined by orthophosphate analysis,[17] after hydrolysis of aliquots with 70 per cent $HClO_4$ at 170°C.[18] The lipid classes were separated by thin layer chromatography on plates of silica gel H. The separated components were localized by spraying with molybdate acid reagent.[19] Ninhydrin was used to identify the aminophospholipids (phosphatidylserine and phosphatidylethanolamine). Quantitation of lipid classes was performed in scraped spots by measuring orthophosphate as described above.

Liposomes of soybean phosphatidylcholine were prepared according to Dinis *et al.*[20] Unilamellar

liposomes were prepared by the extrusion technique as described by MacDonald *et al.*,[21] with seven passes through Avestin polycarbonate membrane 50×, pore 200 nm. Liposomes from mitochondrial lipids were prepared by hydrating a lipid film in a round bottom flask with a buffer containing 10 mM HEPES, 50 mM NaCl, at pH 8·5. The high content of unsaturated cardiolipin species (c.a. 16 mol%) results in serious technical issues when attempting to prepare liposomes at pH 7 or 7·5. Empirically, it was found that the optimal pH is 8·5 which avoids the formation of aggregates rendering a homogeneous preparation.

Evaluation of Lipid Peroxidation

Peroxidation of liposomal lipids has been estimated kinetically from the fluorescence quenching of incorporated *cis*-parinaric acid, as described previously by Dinis *et al.*[20] Peroxidation challenge was induced by suitable mixtures of Fe^{2+}/ascorbate, as described in the legends of the Figures. Oxidation was also elicited by peroxyl radicals generated at a constant rate in the aqueous phase by thermal decomposition of 20 mM AAPH at 37°C and also in the lipid phase from incorporated AMVN (330 μM) at 56°C. Amiodarone effects were evaluated in samples supplemented with the drug (2·5 to 10 μM) added to the liposome suspension or incorporated in lipids during the preparation of liposomes.

Formation of lipid peroxides has been evaluated by measurements of oxygen consumption with a Clark electrode connected to a recorder. Oxidation was induced by thermal decomposition of AAPH in the aqueous phase (10 mM, 37°C) or AMVN in the lipid phase (500 μM, 56°C). Particular conditions are given in the legends to the Figures.

Degradations of the phospholipid acyl chains have been measured by gas–liquid chromatography of samples oxidized by 20 mM AAPH.[20] Samples were treated and handled as described elsewhere[20] and the fatty acid methyl esters were separated on a Varian 2700 gas chromatograph fitted with a BPX70 SGE column (25 m × 0·53 mm), eluted with N_2 at 3 ml min^{-1}. The temperature program was started at 150°C, kept for 5 min and then increased to 220°C at 4°C min^{-1}. Identification was done by comparing the relative retention times with those of authentic standards. Quantitative determinations were performed by integration of peak heights using a suitable program on a computer.

The traces depicted in all the Figures are representative of at least three independent experiments.

RESULTS

Phospholipid Classes of Beef Heart Mitochondrial Lipids

Identified lipid classes are in agreement with Rouser and Fleischer[22] (Table 1), who reported phosphatidylcholine, phosphatidylethanolamine and cardiolipin as major phospholipid classes.

The presence of cardiolipin in significant amounts (c.a. 16 per cent) jeopardizes liposome preparation. Empirically, it was found that the optimal pH for preparation of liposomes of mitochondrial lipids is 8·5, which avoids the formation of aggregates as a consequence of the presence of unsaturated cardiolipin mixed with phosphatidylcholine,[23] rendering a homogeneous preparation. Attempts to prepare liposomes at pH 7 to 7·5 were unsuccessful irrespective of the composition of the dispersion buffer.

Table 1. Phospholipid classes from beef heart mitochondria.

Fatty acid	Per cent
Origin	1·55 ± 0·39
Phosphatidylinositol	3·18 ± 0·39
Phosphatidylserine	4·97 ± 0·68
Phosphatidylcholine	38·29 ± 0·81
Phosphatidylethanolamine	36·17 ± 0·71
Cardiolipin	16·27 ± 0·55

Values are means ± SD of triplicate determinations.

Peroxidation of Liposomes and Amiodarone Effects

The technique of *cis*-parinaric acid (PnA) fluorescence was set up and optimized as described by Dinis *et al.*[20] Figure 2 describes the behaviour of the fluorescent probe as a function of its concentration (Figure 2A) and the lipid concentration (Figure 2B). Saturation of the signal for 1·5 μM PnA is observed at 200 μM lipid. The fluorescence is a linear function of PnA up to 2 μM; above this concentration, linearity is lost as a consequence of self-quenching related with inner filter effects. For beef mitochondrial liposomes, the saturation of PnA fluorescence occurs at about 300 μM phospholipid and the signal increases linearly up to about 1·5 μM PnA (data not shown). These results closely agree with those reported by Gonçalves

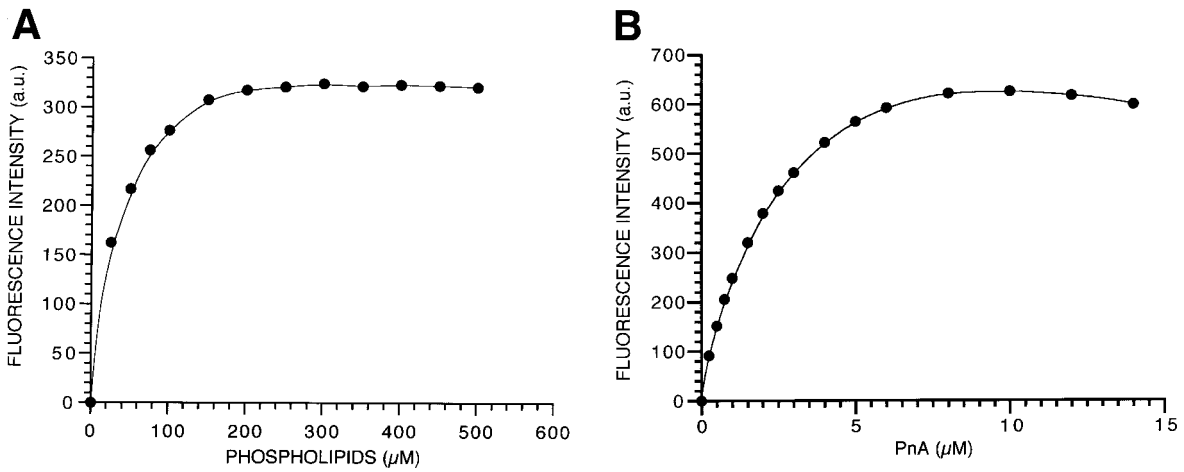

Figure 2. Fluorescence intensity of *cis*-parinaric acid in liposomes as a function of membrane phospholipid and probe concentrations: (A) Parinaric acid (1·5 μM) in 2 ml of buffer (25 mM Tris-HCl pH 7·4, 125 mM KCl) containing different concentrations of soybean PC. (B) Liposomes preparation, equivalent to 200 μM phospholipid, in 2 ml of 25 mM Tris-HCl pH 7·4, 125 mM KCl and different concentrations of parinaric acid added by injection from an ethanolic solution.

et al.[24] for soybean PC lipids, and as described by Van den Berg *et al.*[25] for erythrocyte membranes and Dinis *et al.*[20] for sarcoplasmic reticulum lipids. From these exploratory assays, the operative lipid and probe concentrations have been established as 200 μM and 1·5 μM, respectively.

The effect of amiodarone on soybean PC multilamellar liposomes peroxidation was evaluated by the measurement of oxidative degradation of *cis*-parinaric acid, initiated by addition of Fe^{2+}/ascorbic acid, as described by Dinis *et al.*[20] The optimal combination of initiator/cofator ratio and concentrations were determined from the rate of peroxidative degradation of PnA, by changing the concentrations of Fe^{2+} and ascorbic acid. The course of oxidative degradation of PnA by a mixture of 10 μM ascorbate/10 μM Fe^{2+} was not appreciably altered by amiodarone since a very limited effect that is concentration non-dependent has been detected (Figure 3). This result may be related to the specific distribution of amiodarone that preferentially incorporates into the lipid bilayer core,[26] rather than in regions populated by the lipid segments where oxidation takes place. In order to investigate a potential radical scavenging activity of amiodarone, lipid peroxidation has been induced by thermal decomposition of azoinitiators[27] and the effects were followed by PnA fluorescence quenching (Figure 4A) and oxygen consumption (Figure 4B), as described previously for other compounds of pharmacological interest.[28–31] The addition of water-soluble AAPH generates free radicals in the aqueous phase, whereas lipid soluble AMVN incorporated in multilamellar liposomes generates free radicals within the lipid bilayer.[27,32] When soybean PC unilamellar liposomes are exposed to radicals generated by AAPH, PnA fluorescence rapidly decreases (Figure 4A), and amiodarone is unable to prevent the oxidation of the fluorescent probe. Therefore in spite of the presence of a quaternary ammonium group in the amiodarone structure (Figure 1), this drug is unable to trap radicals generated in the aqueous phase by the thermal decomposition of AAPH; in addition the drug does not prevent propagation of the peroxidation chain in the bilayer. To further ascertain the neutral behaviour of amiodarone as anti- or prooxidant, the lipid peroxidation course has been evaluated by measuring the oxygen consumption related to production of lipid peroxides, as shown in Figure 4B. It is clear that the effects of amiodarone are very limited since the oxygen consumption rate is decreased by 20 per cent at 10 μM amiodarone.

Since amiodarone is very lipophilic, its inability to trap radicals generated in the aqueous phase is expected. To fully address the issue, the azoinitiator AMVN, that undergoes thermal decomposition in the membrane lipid phase with generation of peroxyl radicals, has been used. As shown in Figure 5(A and B), AMVN efficiently induces lipid peroxidation, and amiodarone present for a preincubation period of 5 min before initiation of the peroxidation process, has only a very limited

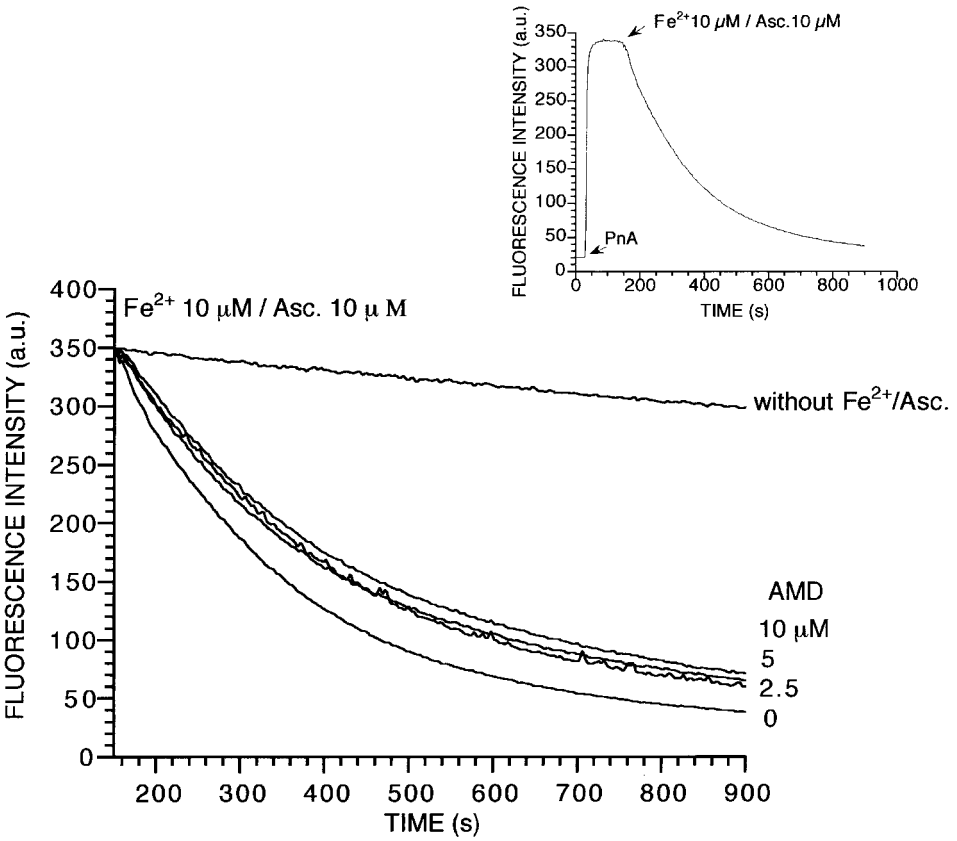

Figure 3. Effect of amiodarone on Fe^{2+}/ascorbic acid induced oxidative degradation of *cis*-parinaric acid incorporated into soybean PC multilamellar liposomes: Different concentrations of amiodarone (2·5, 5·0 and 10·0 μM) were pre-incubated during 5 min in the reaction mixture containing 1·5 μM PnA, liposomes of soybean PC (200 μM in lipid) in 10 mM Na_2HPO_4 and 100 mM NaCl pH 7·4. The lipid peroxidation was initiated by addition of Fe^{2+}/ascorbic acid (10 μM/10 μM). The fluorescence decay in presence of amiodarone was compared with the control assay (absence of amiodarone). (Insert) Oxidative degradation of PnA (1·5 μM) induced by Fe^{2+}/ascorbic acid (10 μM/10 μM) in multilamellar liposomes (200 μM). The fluorescence intensity was recorded with time.

antioxidant effect, as can be seen by the results shown in Figures 4B and 5B. These data reinforce the conclusions that amiodarone does not afford protection against peroxidation of the lipid bilayer nor does it induce any potentiation of the process.

Peroxidation of Mitochondrial Lipids and Amiodarone Interaction

Figure 6A shows the effect of Fe^{2+}/ascorbic acid as a peroxidation inducer system. It can be seen that the rate of peroxidative degradation of PnA in liposomes of lipids extracted from beef heart mitochondria depends on the initiator/cofator ratio and concentrations. Addition of Fe^{2+} in a system with ascorbic acid (20 μM) increases degradation of PnA as a function of Fe^{2+} concentration, similar to reports for sarcoplasmic reticulum membranes.[20]

After ascertaining the optimal concentrations and Fe^{2+}/ascorbic acid ratios, amiodarone was assayed for a putative effect either to revert or to increase the peroxidation rate. Data of Figure 6B clearly show that the drug does not change the initial stages of lipid peroxidation, but it decreases by about 30 per cent the amount of oxidized PnA at the end of the assay. Apparently, the effects are not concentration dependent since the differences observed for 2·5 and 10 μM amiodarone are small. As in the case of soybean lipids, AAPH has also been used owing to its ability to generate radicals in the aqueous phase at a constant rate, by thermal

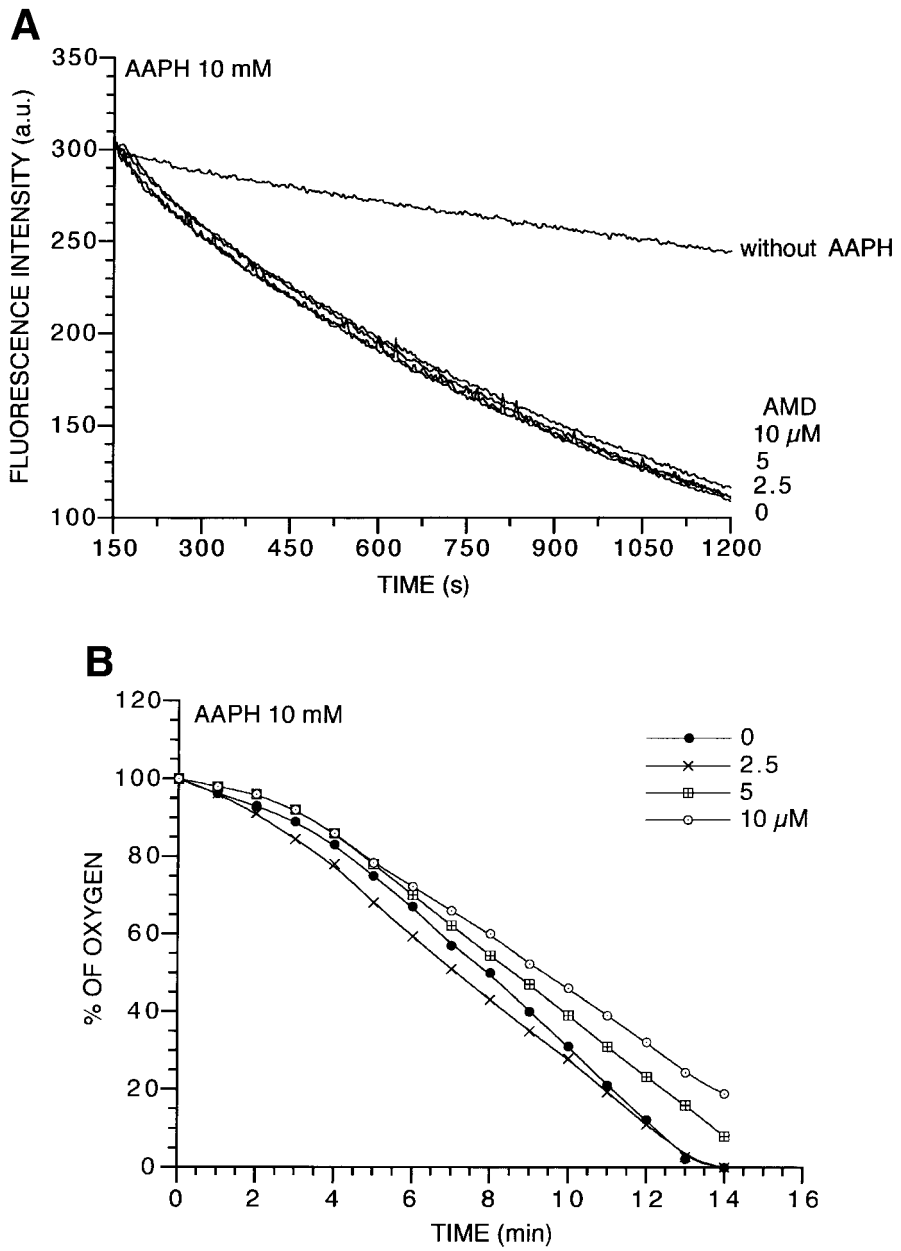

Figure 4. Effect of amiodarone on lipid peroxidation induced by peroxyl radicals generated from thermal decomposition of AAPH, in soybean PC liposomes: (A) Amiodarone (2·5, 5 and 10 μM) was pre-incubated for 5 min with unilamellar liposomes of soybean PC (200 μM phospholipid); PnA (1·5 μM) was injected into 2 ml of liposome suspension and the peroxidative degradation was induced by 10 mM AAPH, at 37°C. (B) Rates of oxygen consumption during oxidation of unilamellar liposomes of soybean PC induced by 10 mM AAPH at 37°C in the absence or the presence of amiodarone (2·5, 5 and 10 μM). The drug was pre-incubated with unilamellar liposomes for 5 min.

decomposition. The fluorescence decay of PnA following the addition of AAPH reflects its oxidative degradation, as previously demonstrated.[20] Incorporation of amiodarone in liposomes, before addition of AAPH, slightly slows down the fluorescence decay of PnA relative to the control assay (Figure 7), in agreement with data of Fe^{2+}/ascorbate (Figure 6B).

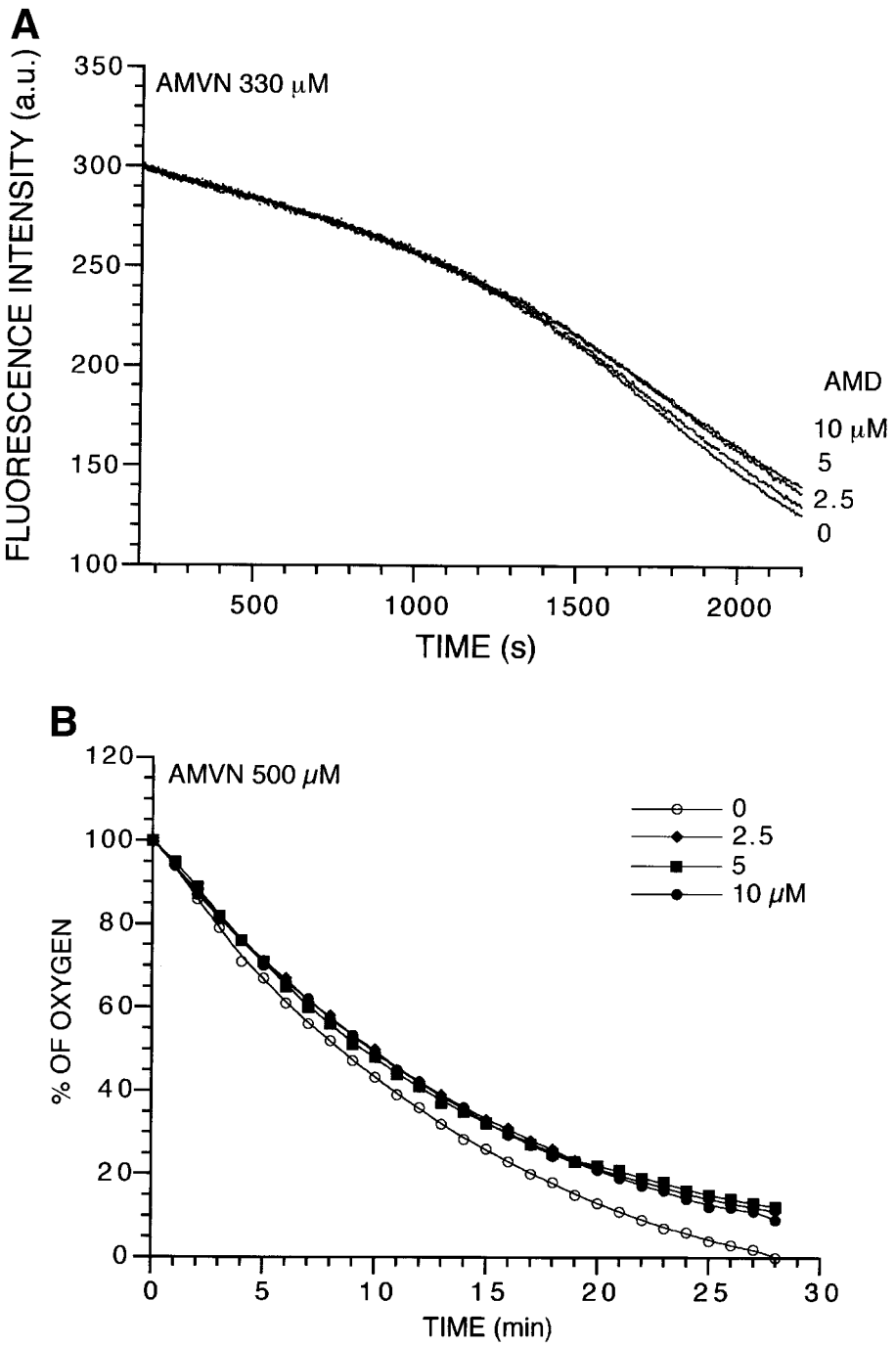

Figure 5. Effect of amiodarone on lipid peroxidation induced by peroxyl radicals from thermal decomposition of AMVN incorporated in soybean PC liposomes: (A) The reaction mixture contained 7·5 μM PnA, 330 μM AMVN incorporated in multilamellar liposomes of soybean PC (800 μM in lipid), 10 mM Na_2HPO_4 and 100 mM NaCl pH 7·4 at 56°C, in a final volume of 2 ml. Amiodarone (2·5, 5 and 10 μM) was added into the system before PnA incorporation. (B) Rates of oxygen consumption induced by 330 μM AMVN incorporated in multilamellar liposomes of soybean PC (800 μM). The reaction mixture contained 10 mM Na_2HPO_4 and 100 mM NaCl pH 7·4, at 56°C in a final volume of 1 ml. Amiodarone (2·5, 5 and 10 μM) was added at the beginning of the oxidation.

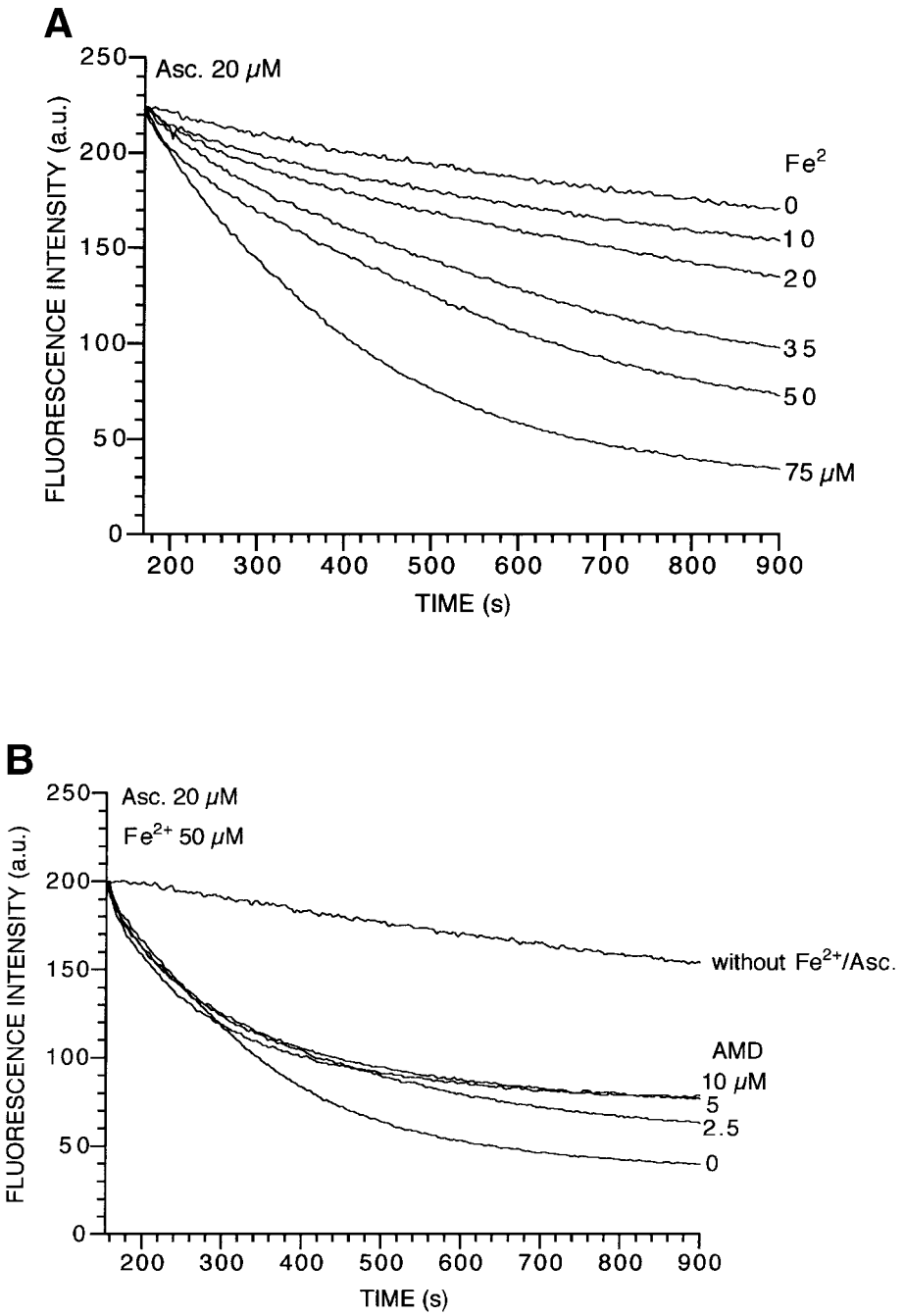

Figure 6. Effect of amiodarone on oxidative degradation of *cis*-parinaric acid incorporated into liposomes of lipids from beef heart mitochondria induced by Fe^{2+}/ascorbate: (A) Peroxidative degradation of PnA incorporated into liposomes of mitochondrial lipids induced by Fe^{2+} in presence of 20 μM ascorbic acid. PnA (1·5 μM) was injected into 2 ml of liposomes suspension (200 μM phospholipid) and fluorescence intensity was recorded with time. (B) Amiodarone (2·5, 5 and 10 μM) was incorporated in multilamellar liposomes of mitochondrial lipids, and the lipid peroxidation was induced by addition of Fe^{2+}/ascorbic acid (50 μM/20 μM). The reaction mixture contained 1·5 μM PnA, liposomes of mitochondrial lipids (200 μM in lipid), 10 mM HEPES, 50 mM NaCl, pH 8·5, in a volume of 2 ml, at 25°C. The fluorescence decay, in presence of amiodarone, was compared with the control assay (absence of amiodarone).

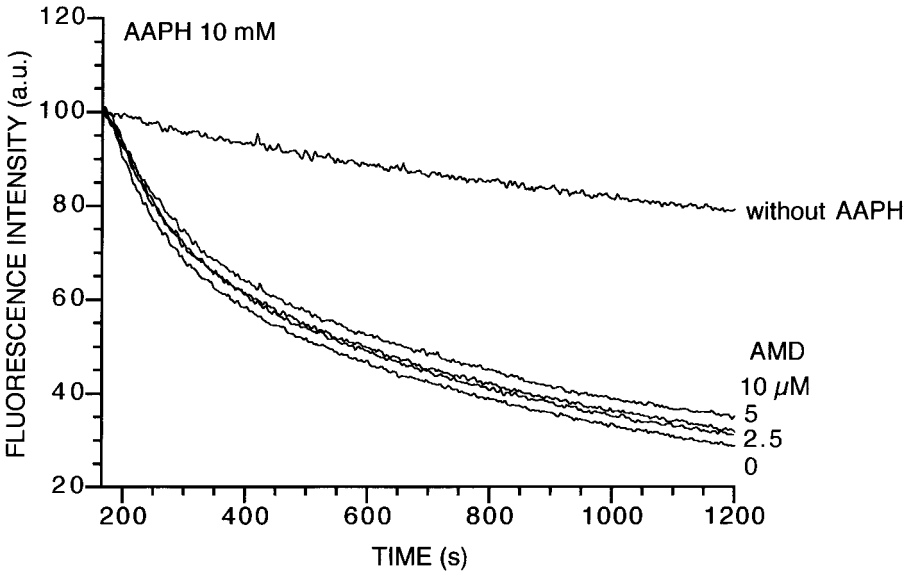

Figure 7. Effect of amiodarone on the oxidative degradation of *cis*-parinaric acid incorporated into liposomes of beef heart mitochondrial lipids induced by thermal decomposition of AAPH: PnA oxidation rates in multilamellar liposomes from mitochondrial lipids, induced by AAPH (10 mM), in the absence or the presence of amiodarone. This drug was incorporated into liposomes from mitochondrial lipids. The reaction mixture contained 1·5 μM PnA, 200 μM phospholipids from liposomes of mitochondrial lipids, 10 mM HEPES pH 8·5 and 50 mM NaCl in a volume of 2 ml, at 37°C.

Degradation of Acyl Chains of Phospholipids

The PnA methodology that records the degradation of the unsaturated chain of PnA putatively reflecting peroxidation of unsaturated chains of phospholipids[13] is complementary to the oxygen consumption which reflects the formation of lipid peroxides. These useful kinetic techniques have been complemented by a direct measurement of phospholipid acyl chain degradation, by gas–liquid chromatography of fatty acyl chain methyl esters, to evaluate the effect of amiodarone on the true degradation and breakdown of the aliphatic chains. Table 2 shows that incubation of liposomes for 1 h in the presence of AAPH (20 mM) at 37°C decreases the relative amounts of PUFA (polyunsaturated fatty acids) with a consequent relative increase in the amount of saturated and

Table 2. Phospholipid fatty acyl composition of liposomes from beef heart mitochondria (control) expressed as a percentage of total fatty acyl chains analysed and after 1 h of peroxidation induced by AAPH 20 mM at 37°C, in the absence or the presence of 5 μ^m and 10 μ^m amiodarone, monitored by gas–liquid chromatography.

	Control	No drug	AMD 5 μ^m	AMD 10 μ^m
16:0 ald	7·89 ± 0·16	7·58 ± 0·19	7·71 ± 0·27	7·97 ± 0·05
16:0	11·12 ± 0·32	13·19 ± 0·27	11·89 ± 0·53	13·04 ± 0·48
18:0 ald	4·04 ± 0·58	4·11 ± 0·19	4·36 ± 0·19	4·43 ± 0·06
18:0	10·10 ± 0·15	21·04 ± 0·27	20·72 ± 0·23	20·70 ± 0·20
18:1	17·47 ± 0·36	18·85 ± 0·36	18·85 ± 0·37	19·06 ± 0·15
18:2	16·73 ± 0·18	16·96 ± 0·15	17·73 ± 0·23	17·22 ± 0·18
18:3	3·81 ± 0·04	3·44 ± 0·06	3·61 ± 0·20	3·48 ± 0·05
20:4*	12·21 ± 0·20	9·59 ± 0·24	10·48 ± 0·40	9·27 ± 0·57
20:5*	4·30 ± 0·09	2·90 ± 0·02	3·25 ± 0·14	2·79 ± 0·25
22:5*	2·57 ± 0·10	2·26 ± 0·25	2·37 ± 0·11	1·93 ± 0·16
22:6*	0·35 ± 0·02	0·14 ± 0·01	0·21 ± 0·03	0·19 ± 0·02

Values are means ± SD of triplicate determinations.
*Fatty acids that undergo more extensive degradation.
ald stands for dimethylacetal chains of plasmalogenic phospholipid species.

mono-unsaturated fatty acids. The most significant changes were observed for PUFA with four or more double bonds: arachidonic (20:4), eicosopentaenoic (20:5), docosopentaenoic (22:5) and docosohexaenoic (22:6) acids. The less unsaturated linoleic (18:2) and linolenic (18:3) acids are not degraded to a significant extent, as also observed with lipids of sarcoplasmic reticulum.[20] Incubation with amiodarone slightly decreases the oxidative degradation of PUFA in agreement with the limited effects detected by the kinetic techniques (Figures 4B and 5B).

Therefore it can be definitely concluded that amiodarone does not exert any significant effects upon lipid peroxidation either as antioxidant or prooxidant. It must be pointed out that antioxidant effects do not necessarily mean affordable protection against oxidative degradation since the radicals resulting from 'antioxidant' chemicals can be of increased reactivity relative to the radicals trapped by the so-called 'antioxidant' compounds. Therefore, a silent action of amiodarone in oxidative processes can be advantageous in its therapeutic use, since side toxicity effects related with lipid peroxidation can be ruled out.

DISCUSSION

Amiodarone is a highly effective antiarrhythmic agent, but its therapeutic use is associated with a variety of side-effects involving many different organs and tissues. The mechanism of toxicity is multifactorial and may result from the accumulation of substances including amiodarone itself, altered cellular function, the development of cellular phospholipidosis secondary to phospholipase inhibition and the formation of free radicals.[33] It has been shown here that amiodarone is unable to affect the lipid peroxidation in liposomes of soybean PC and in liposomes from beef heart mitochondrial lipids.

These results are in contrast with data of Rekka *et al.*[11] and Ribeiro *et al.*[12] who have reported that amiodarone may inhibit non-enzymic lipid peroxidation in inactive rat hepatic microsomal fractions and rat liver mitochondria, respectively. Their assays were performed by measuring thiobarbituric acid-reactive substances (TBARS), not directly related with lipid degradation following peroxidation. Actually, most of the malondialdehyde (MDA) measured may not be formed during peroxidation itself, but during formation and breakdown of lipid peroxides in the acid-heating process, especially in the presence of metal catalysts. Also, the amount and type of acid used in the TBARS assay may influence the TBA-reactive material formed.[13]

In our work, the lipid peroxidation has been monitored by using more specific methods, rather than TBARS, better to elucidate the interaction of amiodarone previously described as an antioxidant by Rekka *et al.*[11] The lipid peroxidation was measured by the oxidative degradation of PnA, oxygen consumption and by gas–liquid chromatography of fatty acyl chain methyl esters. The use of the fluorescent probe *cis*-parinaric acid (PnA) is appropriate to monitor the initial stages of lipid peroxidation. It offers several advantages over other methods since it is direct, sensitive and, therefore, useful to monitor kinetically the susceptibility of membranes to lipid peroxidation.[25,28] The method may be a good alternative over other methods because PnA fluorescence quenching can be followed continuously without destruction of the sample.[34]

The measurement of oxygen consumption during the propagation steps of lipid peroxidation also allows the continuous recording of lipid peroxidation and although it is indirectly related to the peroxidation,[34] it confirms the *cis*-parinaric data.

Amiodarone interaction has been assayed in two different models of lipid membranes. Initially, the lipid peroxidation was measured in soybean PC liposomes and the oxidation was induced by iron-dependent and iron-independent systems, i.e. Fe^{2+}/ascorbic acid and the azocompounds AAPH and AMVN, respectively, in the absence or the presence of amiodarone. Iron is particularly effective as a catalyst of lipid peroxidation; as a transition metal, it reacts with oxygen to form species capable of initiating oxidative degradation or reacts directly with lipid peroxides propagating the reaction.[35] These assays were performed by PnA fluorescence quenching and oxygen consumption. In all situations, amiodarone does not significantly alter the oxidation profiles, whether the radical generation is activated in the aqueous or the lipid phase of model membranes. The lipid peroxidation has also been evaluated in liposomes of lipids extracted from beef heart mitochondria. The oxidations induced by Fe^{2+}/ascorbic acid and AAPH monitored by PnA fluorescence quenching are almost insensitive to amiodarone, even when the drug is incorporated during the preparation of liposomes (Figure 7). Therefore, amiodarone is neutral in inhibiting Fe^{2+}/ascorbate induced lipid peroxidation as well

as in scavenging peroxyl radicals generated either in the aqueous phase or in the lipophilic domains of the membrane. Possible artifacts and misinterpretations were ruled out by evaluating the degradation of fatty acyl chains of phospholipids monitored by gas–liquid chromatography, a reliable and specific technique for monitoring the oxidative degradation process.[13] Data obtained by GLC essentially confirm that amiodarone does not exert any significant effects, either as an antioxidant or prooxidant.

According to our experiments, non-significant differences of amiodarone effects were noticed in liposomes of soybean PC or beef heart mitochondrial lipids, in agreement with Chatelein and Laruell[36] who reported similar amiodarone incorporations in different lipid systems as a consequence of a strong partition coefficient (ca. 10^6).

Further studies in submitochondrial particles are currently underway to ascertain if the drug can alter lipid peroxidation of the native membranes in relation to the inhibition of the mitochondrial redox complexes promoted by the drug.[37,38]

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