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The induction of pulmonary phospholipidosis and the inhibition of lysosomal phospholipases by amiodarone

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Summary. Administration of high doses of amiodarone to young adult rats leads to phospholipidosis of the lung, with extensive phospholipid storage by type II pneumonocytes and alveolar macrophages. Biochemical analysis reveals an increase in the total phospholipid content of the lung and in the proportion of phosphatidylcholine. The cause of the phospholipidosis is suggested to be the inhibition of lysosomal phospholipases, responsible for catabolising phospholipids. It is shown that amiodarone is a potent inhibitor of phospholipases prepared from the soluble fraction of adult rabbit lung lysosomes.

Keywords: lung, phospholipidases, lysosomes, amiodarone, phospholipidosis

Amiodarone (Cordarone X) is a cationic amphiphilic drug, 2-butyl-3-(3,5-diiodo-4-(2-diethylamino)ethoxy)benzofuran. As the hydrochloride, it is used very effectively as an anti-arrhythmic. Side-effects include corneal and skin microdeposition of lipofuscin, suggesting an interference with the lysosomal system. Phospholipidosis of the liver has been reported (Poucell *et al.* 1984), with lamellar lysosomal inclusions in liver cells. Peripheral neuropathy induced by the drug is associated with the presence of lysosomal inclusions in Schwann cells, and endothelial and perineural cells (Dudognon *et al.* 1979). Pulmonary problems also occur, particularly with high-maintenance-dose regimes, and present as interstitial changes, sometimes progressing to alveolitis (McKenna *et al.* 1983). Histological examination reveals foamy alveolar macrophages, hyperplastic type II pneumonocytes and widened alveolar septa. The cells show granular and lamellar structures within distended lysosomes (Marchlinski *et al.* 1982). The lung is particularly susceptible to phospholipidosis since the type II epithelial cells synthesize and secrete pulmonary surfactant, a material rich in phosphatidylcholine. Similar phospholipidoses have been found as side-effects of other cationic amphiphilic drugs (Lüllmann-Rauch 1979). The effects of chlorphentermine, chlorocyclizine, imipramine, chloroquine and chlorimipramine on rat lung have recently been described in detail (Reasor & Heyneman 1983; Stern *et al.* 1983; Gräbner & Meerbach 1983; Sgaragli *et al.* 1983). These studies suggest that inhibition of lysosomal phospholipases is the cause of the phospholipidosis, an idea supported by the finding that many of the drugs are powerful inhibitors *in vitro* (Hostetler & Matsuzawa 1981). Experimental treatment of rats and mice with amiodarone leads to phospholipid accumulation in alveolar macrophages and type II cells of the lung (Costa-Jussà *et al.* 1984), in certain parts of the

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nervous system (Jacobs & Costa-Jussà 1983), and in other organs (Bockhardt *et al.* 1978). We have therefore examined the cellular and biochemical changes in treated rat lung, and the effect of amiodarone hydrochloride on phospholipases A_1 and A_2 from lysosomes of adult rabbit lung.

Methods

Amiodarone administration. Young adult Sprague–Dawley rats were given amiodarone (kindly supplied by Sanofi UK Ltd) (400 mg/kg/day, suspended in 5% methylcellulose) by oesophageal cannula, for 1 month. Control animals received the vehicle only. The lungs of some animals were perfusion-fixed after inducing deep anaesthesia with pentobarbitone (Sagatal); the lungs of others were removed unfixed and stored frozen for phospholipid analysis.

Light microscopy. After perfusion fixation with glutaraldehyde, pieces of rat lung were treated with osmium tetroxide, dehydrated, and embedded in plastic. Sections of 1 μm were stained with toluidine blue. Post-fixation in osmium tetroxide preserves the phospholipid-containing inclusion bodies. These bodies are not retained during processing for conventional paraffin histology.

Phospholipid analysis. Rat lung tissue samples were weighed and homogenized by hand using a modified Folch procedure (Christie 1973) with methanol (10 vols/g tissue), followed by chloroform (20 vols), and then three further washes with chloroform (10 vols). The phases were separated by centrifugation, and the chloroform layers combined and taken to dryness. The lipids were finally dissolved in chloroform (2 ml) with antioxidant (butylated hydroxytoluene, 0.001%).

A small aliquot (1.25%) was used for total phospholipid-phosphorus estimation by spotting on silica, scraping off, digesting with acid (Hodge 1973) and determining phosphate spectrophotometrically with malachite green (Anner & Moosmayer 1975). Individual phospholipids were determined using a similar aliquot of the extract, but after separation by two-dimensional thin-layer chromatography (TLC). This was carried out using Merck silica gel 60 TLC plastic sheets which had been cut into 10 × 10 cm plates. The solvents used were: dimension 1, chloroform:methanol:ammonia, SG 0.880 (63:35:5, by vol.); dimension 2, chloroform:methanol:water (65:35:4), by vol.). The TLC sheets were then stained with iodine vapour, the spots identified by comparison with standard phospholipids, and then estimated spectrophotometrically as before. There was always a small proportion of phosphorus-containing spots which could not be identified, and these are included for purposes of percentage calculation.

Enzyme preparations. Adult New Zealand White rabbits were killed by intravenous barbiturate. The lung vasculature was then perfused *in situ* with a solution of balanced salts and glucose via the pulmonary artery while ventilation was performed manually through a tracheal cannula. When free of blood, the lungs were excised and lavaged five times with balanced salts/glucose. After homogenization of the lungs in 0.25 M sucrose in 0.05 M Tris-HCl, pH 7.0, the soluble subfraction of lysosomes was obtained as previously described (Heath & Jacobson 1984*a*,*b*). Pulmonary macrophages were recovered from the lavage fluid by low-speed centrifugation, and the cell pellet was disrupted by five cycles of freezing and thawing.

Enzyme assays. Lysosomal phospholipases A_1 and A_2 were assayed at pH 4.0 in the presence of 5 mM Na_2EDTA as previously described (Heath & Jacobson 1984*a*,*b*), except that the concentration of the substrate, 1-palmitoyl 2-[^{14}C]oleoyl phosphatidylcholine, was increased to 100 μM. Amiodarone hydrochloride was added to the incubation, as necessary, dissolved in 0.08 M sodium acetate, pH 4.0. Comparative inhibition studies were also undertaken with im-

ipramine and chloroquine (the latter at pH 5·5).

Results and discussion

Cellular changes

The two most striking cellular changes found in adult rat lung after exposure to high doses of amiodarone affect the type II pneumonocytes, which synthesize pulmonary surfactant, and macrophages, whether in the alveolar lumina, the alveolar septa or the interstitial connective tissue.

Changes in type II cells. In the lungs of control animals, type II cells contain only a few osmiophilic lamellated granules of 1 to 2 μm diameter, while type II cells of amiodarone-treated rats are laden with 10, 20 or more of these cytoplasmic inclusions of greatly varying size (5 μm or even more in diameter), thus causing a marked enlargement of the individual cell. In some of the cells the presence of many, or large, inclusions may cause indentation of the nucleus. Occasionally, a cell can be found in the process of discharging particles into the alveolar lumen, where free particles of this material can also be seen (Figs 1 and 2). The number of type II cells lining an alveolus is not significantly greater than in control animals, and a careful search did not reveal an increased mitotic rate. The consequences of the great increase in volume of the individual cell are threefold: (i) parts of the alveolar wall

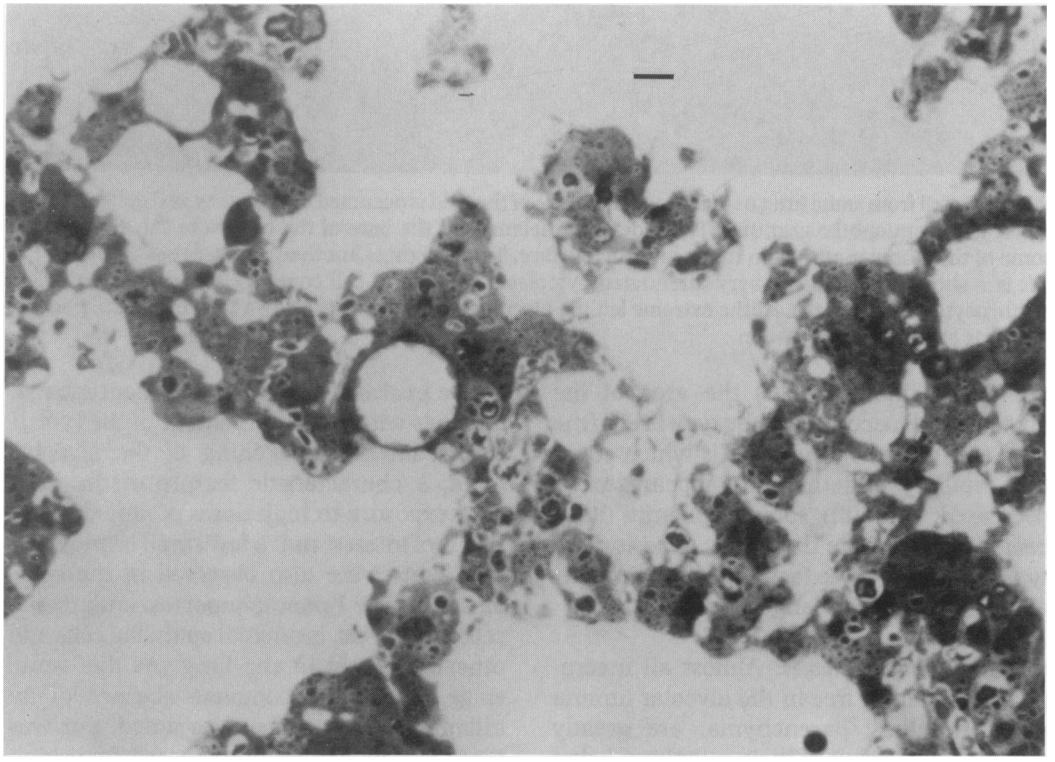

Fig. 1. 1-μm-thick section of lung from a rat that received 400 mg amiodarone/kg/day, by mouth, for 1 month. (Glutaraldehyde, OsO_4, Epon, Toluidine blue.) The section shows a large number of type II alveolar epithelial cells laden with osmiophilic granules of varying sizes. The type II cells occupy an increased alveolar surface area, as is apparent from following the outlines of the airspaces. Only relatively short stretches of alveolar lining are covered by the thin cytoplasm of type I cells. Note the thickened alveolar septa, due to the large size of the swollen type II cells. Bar = 10 μm.

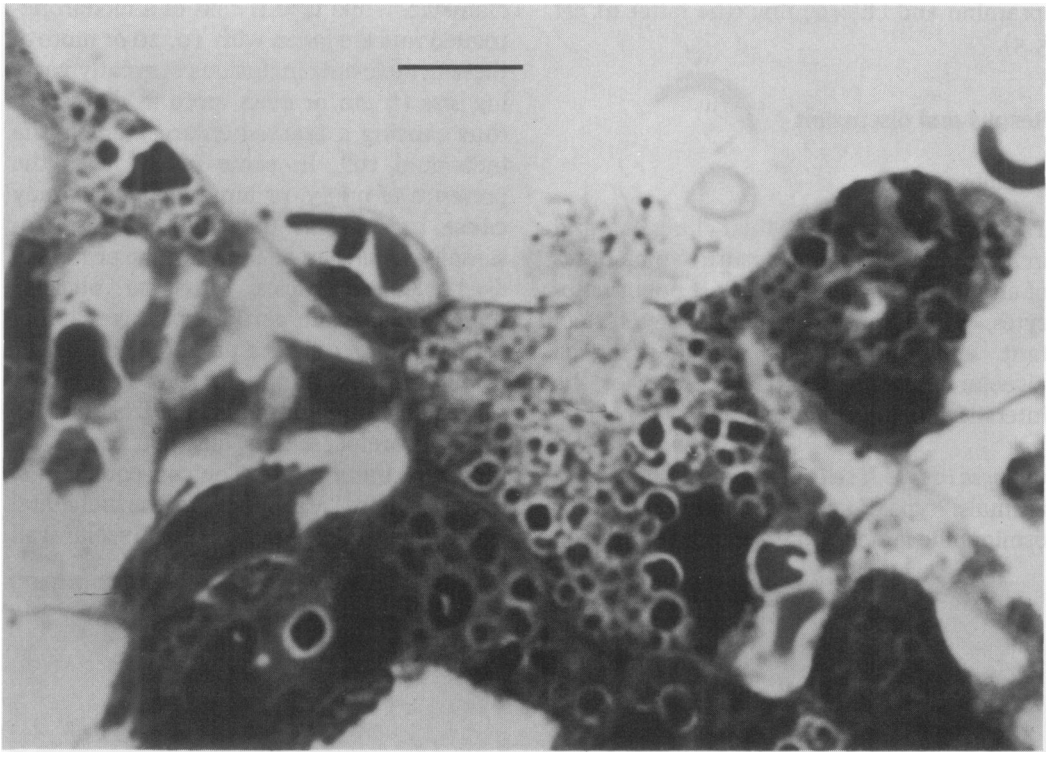

Fig. 2. Detail from same lung as in Fig. 1. The centre of the field is occupied by a large type II epithelial cell, laden with osmiophilic granules. The indented nucleus is at the base of the cell. Note the discharge of some of the stored material on to the alveolar surface. To the right is another, smaller type II cell. To the left is a short stretch of the very attenuated cytoplasm of a type I cell covering a capillary (with two erythrocytes in its lumen). At the extreme left the alveolar surface is covered by a type II cell, only part of which is shown. Bar = 10 μm.

are much thickened; (ii) the area of the alveolar wall occupied by the type I cells (the very attenuated epithelial cells which cover the capillary endothelium) appears to be decreased; and (iii) some capillaries have been dislodged from their close apposition to type I cells. All three factors would diminish an effective gas exchange.

Changes in macrophages. Almost all macrophages, whether free in the alveolar lumina or in the lung parenchyma, are greatly enlarged as a result of their cytoplasm being laden with ingested material in the form of inclusion bodies, many of them lamellated. This indicates that their phagocytic capacity has continued while their catabolic activity has been severely impaired. The great increase in size of the individual macrophage, together with the enlargement of the type II cells, causes a thickening of the alveolar septa, a characteristic feature of the lung after exposure to high doses of amiodarone.

It is of interest that a few small osmiophilic inclusions were also observed in the cytoplasm of type I pneumonocytes, endothelial cells, fibrocytes, bronchial epithelial cells and other cell types in the lung. At this acute stage, an almost complete absence of an inflammatory response was noted, nor was there any indication of increased deposition of collagen fibres.

Phospholipid analysis

The results from the five samples examined,

Table 1. Phospholipid content and composition of lung homogenate samples from control and amiodarone-treated rats

	Control				Amiodarone-treated		
	1	2	3	Mean	4	5	Mean
Total phospholipid (μg P/g tissue)	748.60	379.30	380.10	502.70	2240.50	2448.20	2344.40
Composition (%)							
LPC	4.74	2.81	4.78	4.11	1.82	1.60	1.71
SM	11.45	11.16	7.81	10.14	3.54	3.19	3.31
PC	53.70	50.70	55.08	53.16	74.42	74.81	74.62
PE	18.76	25.57	19.82	21.38	10.72	10.58	10.65
PG	0.00	0.33	1.08	0.69	0.37	0.41	0.39
PA/PI/PS	11.35	9.43	10.34	10.37	5.29	7.72	6.51
Other	0.00	0.00	1.08	0.36	3.74	2.01	2.88

LPC, Lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine.

three controls and two amiodarone-treated samples, are illustrated in Table 1, which shows the total phospholipid content and percentage phospholipid composition together with the mean values for the two groups. Comparison of these shows that treatment with amiodarone produces the following effects: (1) an increase in total phospholipid (about four-fold); (2) a higher proportion of phosphatidylcholine (PC) (53% to 74%), and hence a large absolute increase in the amount of this phospholipid, and (3) reduced proportions of the other phospholipids, which could be interpreted as reflecting the increase in PC.

Enzyme assays

The increased amounts of phospholipid in the lungs of treated rats, and the cellular changes, particularly the loading of the macrophages, suggest that amiodarone is inhibiting the activity of lysosomal phospholipases, enzymes responsible for the catabolism of phospholipid. Table 2 shows the inhibitory effect of 50 μM amiodarone on lysosomal phospholipases A_1 and A_2 from rabbit lung parenchyma and in rabbit alveolar macrophages. The residual phospholipase activities are higher in the macrophages, though the difference is not significant (Student's *t*-test). Any real difference could be due to non-lysosomal enzymes present in these whole-cell preparations. Fig. 3 shows the effect of increasing amiodarone concentration on a typical extract from the lysosomal fraction of adult rabbit lung. The apparent inhibitor concentration for half inhibition (IC_{50}) is 7 μM. This is one of the lowest values yet reported for a cationic amphiphilic drug (Matsuzawa & Hostetler 1980; Hostetler & Matsuzawa 1981; Stern *et al.* 1983), indicating a powerful inhibitor. In our system, the IC_{50} for imipramine is 110 μM, and while chloroquine is ineffective at pH 4.0, it registers an IC_{50} of 410 μM at pH 5.5 (cf. Matsuzawa & Hostetler 1980). The plasma concentration of amiodarone for patients on a normal maintenance dose regime is 1 to 2 μM (McKenna *et al.* 1983), but the tissue concentrations are probably higher. Like any weak organic base, amiodarone will concentrate in the acid interior of lysosomes, reaching tissue concentrations 100- to 1000-fold higher than in plasma, as has been shown for chlorimipramine

Table 2. Residual activities of lysosomal phospholipases from rabbit lung in the presence of 50 μM amiodarone hydrochloride

Source	Activity (% of control) Phospholipase A_1	Phospholipase A_2
Soluble lysosomal extracts (mean ± SEM, $n = 5$)	9.2 ± 2.0	3.4 ± 2.5
Pulmonary macrophages (frozen/thawed × 5) (mean ± SEM, $n = 3$)	16.0 ± 6.7	6.0 ± 1.2

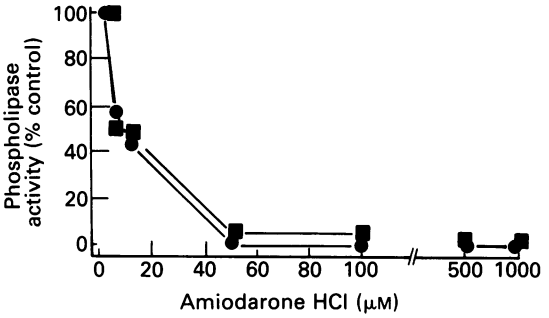

Fig. 3. The effect of increasing concentrations of amiodarone hydrochloride on the hydrolysis of phosphatidylcholine by a soluble extract of the lysosomal fraction from adult rabbit lung. The residual activities of phospholipase A_1 (■) and phospholipase A_2 (●) are shown as a percentage of the activities in the absence of amiodarone.

(Sgaragli *et al.* 1983). At these levels, the phospholipase activity of the lysosomes will be very seriously inhibited, so that in the lung, which secretes a large amount of phospholipid as pulmonary surfactant, a phospholipidosis will readily develop.

Conclusions

Amiodarone treatment of young adult rats leads to marked cellular changes in the lung. Abundant lamellar material is found in the type II pneumonocytes, in the alveolar lumina, and particularly filling the lysosomes of the macrophages. Biochemical analysis shows a four-fold increase in the phospholipid content of the lung, much of it due to increased amounts of phosphatidylcholine, the major component of pulmonary surfactant. Studies *in vitro* indicate that amiodarone is a powerful inhibitor of the lysosomal phospholipases from adult rabbit lung, and we suggest that this block of phospholipid catabolism is the primary cause of the phospholipidosis. Phospholipase A_2 may also be involved in the synthesis of the principal molecular species of PC in pulmonary surfactant, dipalmitoyl phosphatidylcholine, via a remodelling process (discussed by Heath & Jacobson 1984*a*,*b*). The inhibition of this enzyme might therefore also result in the production of a surfactant of lower quality, and this may exacerbate the pulmonary problems.

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