Amiodarone inhibits lung degradation of SP-A and perturbs the distribution of lysosomal enzymes

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Amiodarone has been shown to induce lung damage by direct toxicity or indirectly through inflammation. To clarify the mechanism of direct toxicity, we briefly exposed rabbit alveolar macrophages to amiodarone and analyzed their morphology, synthesis, and degradation of dipalmitoylphosphatidylcholine (DPPC); distribution of lysosomal enzymes; and uptake of diphtheria toxin and surfactant protein (SP) A used as tracers of the endocytic pathway. Furthermore, in newborn rabbits, we studied the clearance of DPPC and SP A instilled into the trachea together with increasing amounts of amiodarone. We found that in vitro amiodarone decreases the surface density of mitochondria and lysosomes while increasing the surface density of inclusion bodies, increases the incorporation of choline into DPPC, modifies the distribution of lysosomal enzymes, and does not affect the uptake and processing of diphtheria toxin but inhibits the degradation of SP-A. In vivo amiodarone inhibits the degradation of SP-A but not of DPPC. We conclude that the acute exposure to amiodarone perturbs the endocytic pathway acting after the early endosomes, alters the traffic of lysosomal enzymes, and interferes with the turnover of SP-A.

Morphologically, the damage to the lungs is characterized by the accumulation of multilamellar bodies in the cytoplasm of various cell types and by inflammation. In some patients, the inflammatory changes lead to fibrosis (18, 30).

The accumulation of multilamellar bodies in the cytoplasm has been extensively studied and is thought to be due to a decreased degradation of phospholipids because amiodarone is a powerful inhibitor of lysosomal phospholipases A1 and A2 (14, 16, 19, 24). The origin of the inflammation, on the other hand, is less clear, and the literature presents both studies supporting and studies refuting a role for the immune system in the genesis of lung damage (reviewed in Ref. 31). Studies supporting a role for the immune system (17, 32, 33, 37) have shown that amiodarone can activate lung natural killer cells and change the pattern of secretion of cytokines by alveolar macrophages, but it is unclear if the observed changes represent the starting lesion or are the consequence of direct lung damage. In fact, amiodarone could induce direct damage by accumulating in lysosomes and disturbing crucial catabolic processes, changing the physical properties of cell membranes, inhibiting mitochondrial functions like beta-oxidation, disturbing calcium homeostasis, producing reactive oxidant species, inhibiting ionic pumps, influencing the activity of G proteins or phospholipase C, and inducing apoptosis (1, 5, 16, 23, 31, 36). Some of the changes induced by amiodarone could also be due to interference with the turnover of surfactant because it has been shown that amiodarone causes type II cell hyperplasia and deposition of surfactant protein (SP) A in the alveoli (27).

The aim of this study was to clarify the mechanism of direct lung damage induced by an acute exposure to amiodarone. Thus, using rabbit alveolar macrophages, we examined the effect of amiodarone on the ultrastructure, the incorporation of choline into dipalmitoylphosphatidylcholine (DPPC), the degradation of exogenous DPPC, the distribution of lysosomal hydro-
lases among organelles isolated from the postnuclear supernatant, the release of lysosomal hydrolases into the extracellular milieu, and the targeting of extracellular substrates to the appropriate cellular compartments. The substrates tested were diptheria toxin and SP-A, two proteins that follow different routes after uptake. In fact, diptheria toxin binds to a specific receptor and is delivered to the early endosomes where it is hydrolyzed. From the early endosomes, the active fragment is then translocated to the cytosol where it inhibits protein synthesis through ADP-ribosylation of elongation factor 2 (34). SP-A is taken up by alveolar macrophages and moved to the lysosomes where it is degraded (2). Besides these in vitro studies, we analyzed the clearance of surfactant DPPC and SP-A administered into trachea of 3-day-old rabbits together with increasing amounts of amiodarone.

We found that amiodarone at concentrations that do not affect macrophage viability (1) induces the formation of cytoplasmic vacuoles, increases the surface density of inclusion bodies containing electron-dense material and/or multilamellar membranes, and decreases the surface density of mitochondria and lysosomes; (2) increases the incorporation of choline into DPPC while not affecting the ability to degrade exogenous DPPC; (3) changes the distribution of lysosomal enzymes among cytoplasmic organelles; (4) induces the release into the medium of lysosomal enzymes; and (5) does not affect the uptake, processing, and routing of diptheria toxin but inhibits the degradation of SP-A after uptake. In vivo amiodarone inhibits the degradation of SP-A taken up into tissue compartments but not that of DPPC.

These results indicate that amiodarone perturbs the endocytic pathway acting after the early endosomes and suggest that released lysosomal enzymes may contribute to the genesis of lung damage. They also indicate that the alveolar accumulation of SP-A observed in animals with amiodarone-induced lung toxicity may be due to decreased degradation.

METHODS

Materials. All reagents were of analytic grade. Na1251, 1-O-dipalmitoyl2-palmitoyl-9,10-3H(N)phosphatidylcholine ([3H]DPPC; specific activity 40.0 Ci/mmol), [methyl-3H]choline chloride (specific activity 80 µCi/mmol), and [L-[4,5-3H]leucine (specific activity 151.0 Ci/mmol) were from Amer sham Pharmacia Biotech (Little Chalfont, UK). Before use, [3H]DPPC was purified by preparative thin-layer chromatography. Percoll was from Pharmacia (Uppsala, Sweden). DPPC, egg phosphatidylcholine (PC), egg phosphatidylglycerol, and cholesterol were obtained from Sigma (St. Louis, MO), and their purity was checked by thin-layer chromatography. Amiodarone and desethylamiodarone, stored at −26°C as 100 mM solutions in DMSO, were from Sigma and Sanofi, respectively. Diphereria toxin, kept frozen in liquid nitrogen in 10 mM potassium HEPES-0.1 mM EDTA, pH 7.0, at a concentration of 2 mg/ml, was a kind gift from Prof. Emanuele Papini (University of Padua, Padua, Italy).

Cells. Alveolar macrophages were isolated from bronchoalveolar lavage fluid obtained from adult rabbits. The cells were washed with Ringer buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM Na2HPO4, 10 mM glucose, and 10 mM HEPES, pH 7.4), suspended in Ringer buffer plus 1 mg/ml of bovine serum albumin, 50 IU/ml of penicillin, and 50 µg/ml of streptomycin (RBA), and used either as a suspension or after adhesion to Falcon plates or flasks (Becton Dickinson Lab ware Europe, Meylan, France). We recovered 62.0 ± 3.7 ± 106 (±SE) cells/kg body wt (n = 99 rabbits). The cells were over 90% macrophages (May-Grunwald-Giemsa staining) and 94 ± 1% viable as determined by trypan blue exclusion.

Morphology. Adhering macrophages were fixed overnight in 2.5% glutaraldehyde in PBS, scraped, and centrifuged at 10,000 g for 5 min. The pellets were postfixed for 2 h in 1% osmium tetroxide in PBS, dehydrated, and embedded in Spurr resin. Semithin sections obtained through the whole thickness of the pellets were stained with toluidine blue and observed with a Zeiss Axiohot light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Jeol 1200 EXCII electron microscope.

Morphometry was performed on 10 electron micrographs for each experimental condition (i.e., control and amiodarone-treated cells) randomly taken at ×4,000 magnification and then photographically enlarged to ×10,000 magnification to appreciate the details of at least 2–3 cells/micrograph. A total number of 25 cells in each experimental condition were counted. By means of a ruler inserted within an optical magnifier, we measured the surface area covered by the whole cell and by the mitochondria, lysosomes (i.e., electron-dense, membrane-bound organelles), cytoplasmic inclusions (i.e., electron-dense structures containing amorphous and/or multilamellar membranes, not surrounded by any type of membrane), and endoplasmic reticulum. All organelles that were detectable in the section of each of the randomly selected cells were analyzed. Not less than 100 organelles were analyzed for each experimental condition. Results are expressed as percent of the surface area covered by different organelles in relation to the surface area covered by the whole cytoplasm.

SP-A labeling. SP-A was isolated from rabbit surfactant (3) with the method of Hawgood et al. (13). All preparations of SP-A were tested for lipopolysaccharide contamination with the Limulus amebocyte lysate assay (Limusate, Haemachem, St. Louis, MO), and lipopolysaccharide concentration results were always <0.125 endotoxin unit/ml (<0.2 µg/µg protein). SP-A labeling was done according to Goldstein et al. (12). Briefly, 0.3–0.5 mg of SP-A in 0.6 ml of 5 mM Tris-HCl, pH 7.4, was mixed with 0.2 ml of 1 M glycine, pH 10.0, and then with 500 µCi of 125I. Within 1 min, 0.2 ml of freshly prepared 20 mM ICl was added. After 5 min on ice, the mixture was applied to a PG10 column (Bio-Rad) to separate 125I-SP-A from free iodine. 125I-SP-A was then dialyzed against 5 mM Tris-HCl, pH 7.4, was mixed with 0.2 ml of 1 M glycine, pH 10.0, and then with 500 µCi of 125I. Within 1 min, 0.2 ml of freshly prepared 20 mM ICl was added. After 5 min on ice, the mixture was applied to a PG10 column (Bio-Rad) to separate 125I-SP-A from free iodine. 125I-SP-A was then dialyzed against 5 mM Tris-HCl, pH 7.4. The whole procedure was done at 4°C. 125I-SP-A (specific activity 2–6 × 106 counts·min−1·µg−1) was stored at 4°C and used within 1 mo.

The integrity of labeled SP-A during storage was tested by autoradiography after two-dimensional polyacrylamide gel electrophoresis. Over 99% of the labeled SP-A remained TCA precipitable during storage.

Viability and phospholipid content of alveolar macrophages. To study the effect of amiodarone on viability, alveolar macrophages (106 cells/ml) were cultured for 1 or 24 h as suspensions in the presence of 0–100 µM amiodarone in DMSO (final DMSO concentration 1 µl/ml), and viability was estimated by trypan blue exclusion. In some experiments, after incubation, we pelleted the macrophages, washed them two times with saline, and measured the phospholipids (4) after extraction according to Bligh and Dyer (7). To further...
characterize the effect on viability, macrophages seeded on six-well plates were incubated for 1 h with 0–50 μM amiodarone. The cells were then either collected and homogenized immediately to measure cytochrome-c oxidase (35) or were switched to plain RBA to measure the release of lactate dehydrogenase (LDH) during the next hour (2).

**Choline incorporation by alveolar macrophages.** Macrophages seeded onto six-well plates (2 × 10⁶ cells/well) were incubated for 1 h with 0–50 μM amiodarone in RBA. Without changing the medium, [3H]choline chloride was then added (2 μCi in 100 μl RBA/well), and the incubation was continued for 16 h. At the end, saturated PC associated with the cells was isolated and counted (25). Choline incorporation tended to deviate from linearity during the last 8 h of incubation (data not shown). The cause of the deviation from linearity remains unclear. Less than 5% of added choline was utilized (data not shown). The cause of the deviation from linearity remains unclear. Less than 5% of added choline was utilized during incubation. Results are presented as femtomoles of choline incorporated into DPPC by 10⁶ cells in 16 h (n = 4 experiments).

**Degradation of DPPC by alveolar macrophages.** Suspensions of alveolar macrophages (2 × 10⁶/ml) were incubated for 1 h with 0–100 μM amiodarone and then for 1 more hour with [3H]DPPC (0.05 μCi/ml) presented as liposomes prepared by sonication (2) and with the following composition (in μg/ml): 25 DPPC, 17.5 egg PC, 5 egg phosphatidylglycerol, and 2.5 cholesterol (2). The degradation of DPPC was measured by extracting the incubation mixture (7) and counting the washed water-methanol phase in the presence of Hyonic Fluor (Packard). Control cells degraded 168 ± 24 (SE) ng DPPC-10⁶ cells⁻¹·h⁻¹ (n = 17 experiments). Under the present conditions, the generation of water-soluble by-products was linear up to 100 min (data not shown). The results are presented as percent of the degradation measured with control cells (n = 4 experiments).

**Distribution of lysosomal enzymes in the postnuclear supernatant.** Macrophages seeded onto six-well plates (2 × 10⁶ cells/well) were incubated with 0 or 10 μM amiodarone for 60 min at 37°C, rinsed in 0.25 M sucrose-1 mM EDTA, pH 7.4 (2), scraped, and homogenized in the same medium. The homogenate was centrifuged at 750 g for 7 min to obtain the postnuclear supernatant. Two milliliters of the postnuclear supernatant were deposited over a discontinuous gradient made by a cushion of 2.5 M sucrose in 1 mM EDTA (1.2 ml) and an intermediate layer of 18% Percoll in homogenization medium (8.5 ml) and were centrifuged for 30 min at 20,000 rpm in a Ti 50 rotor (Beckman, Palo Alto, CA) (2). Fractions obtained from the gradient were analyzed for β-hexosaminidase (21) and arylsulfatase (11) activities. The content of each fraction is presented as a percent of the total (n = 4 experiments for β-hexosaminidase and 2 experiments for arylsulfatase).

**Release of lysosomal enzymes by alveolar macrophages.** Alveolar macrophages seeded onto six-well plates (2 × 10⁶ cells/well) were incubated with 0–50 μM amiodarone for 60 min. The medium was then changed with plain RBA, and the incubation was continued for 1 more hour. At the end, the medium was collected and centrifuged at 500 g for 10 min. β-Hexosaminidase activity was then measured in the resulting supernatant and in the cell homogenate (pellet from the 500-g centrifugation plus cells scraped from the plate; n = 11 experiments).

**Effect of amiodarone on the diphertheria toxin-mediated inhibition of protein synthesis.** Alveolar macrophages in RBA were seeded onto 24-well plates (5 × 10⁵ cells/well) and allowed to adhere for 2 h at 37°C. Amiodarone was then added (final concentration 0–50 μM), and the incubation was continued for 1 more hour. Afterward, the medium was substituted with RBA containing 0 or 10 nM diphtheria toxin, and the incubation was continued for 2 h. [3H]leucine (200 nCi/well) was then added for 20 min. At the end, the cells were washed one time with 50% methanol, fixed for 30 min with cold methanol, and then washed three times with cold 10% trichloroacetic acid. The final precipitate was dissolved overnight with 1 N NaOH, and aliquots of the lysate were used for protein assay (29) and to count the radioactivity in the presence of Hyonic Fluor (Packard). Protein synthesis is presented as picomoles of leucine incorporated per milligram of protein in 20 min. Data are from three experiments (29). Control macrophages incorporated 0.82 ± 0.09 (SE) pmol leucine/mg protein in 20 min (n = 7 experiments).

**Association of SP-A with alveolar macrophages.** Suspensions of alveolar macrophages (5 × 10⁶/ml) were incubated for 1 h at 37°C with 0–50 μM amiodarone and then for 0–100 min with 125I-SP-A (1 μg/ml). At the end, the cells were pelleted, transferred to new tubes, and washed two more times with cold RBA before being counted (n = 5 experiments).

**Degradation of SP-A by alveolar macrophages.** Suspensions of macrophages (2 × 10⁶/ml) were incubated for 1 h with 0–100 μM amiodarone, centrifuged, and resuspended in RBA containing 1 μg/ml of 125I-SP-A. After 1 h of incubation, the radioactivity soluble in 20% cold TCA was measured in medium plus cells. Control macrophages degraded 13.6 ± 2.4 (SE) ng SP-A·10⁶ cells⁻¹·h⁻¹ (n = 11 experiments), and the degradation was linearly related with time. Results are presented as percent of degradation measured in the control cells (n = 7 experiments).

**Distribution of SP-A among components of the postnuclear supernatant.** Alveolar macrophages seeded onto six-well plates (2 × 10⁶/well) were incubated for 1 h with 0 or 50 μM amiodarone in RBA and then exposed for 15 min to 1 μg/ml of 125I-SP-A. Afterward, 125I-SP-A was removed, and adhering cells were rinsed three times with RBA. Then the cells were homogenized immediately or were incubated for 30 more minutes in the presence of 1 μg/ml of unlabeled SP-A (chase) before being homogenized. Each homogenate was centrifuged to obtain a postnuclear supernatant that was then centrifuged as described in Distribution of lysosomal enzymes in the postnuclear supernatant over a Percoll-sucrose gradient (2). Fractions collected from the gradient were counted, and the results are expressed as counts per minute per microgram of homogenate DNA (22).

**Clearance of surfactant DPPC and SP-A administered into the trachea.** Labeled surfactant was obtained by administration of 50 μCi of [3H]choline into the tracheae of 3-day-old rabbits and then isolating the surfactant 15 h later as previously reported (3). The surfactant thus obtained was suspended in saline, mixed with 125I-SP-A, and then combined with different amounts of amiodarone dissolved in DMSO. The final mixtures administered to recipient animals contained 5% of the alveolar pool of phospholipids normally found in 3-day-old rabbits, 5 × 10⁴ to 10⁶ dpm as DPPC, 1 μg of 125I-SP-A, and different amounts of amiodarone (0, 2, 10, 50, 250, and 1,250 nmol, corresponding to 0, 1.3, 6.5, 32.3, 161.3, and 806.3 μg and concentrations of 0, 10, 50, 250, 1,250, and 6,250 μM, respectively) suspended in 12.5 μl of DMSO in 200 μl (the volume instilled). The dose was administered to 3-day-old rabbits (5 rabbits for each concentration of amiodarone) by puncturing the trachea that was exposed surgically under local anesthesia (3). After instillation, the animals were returned to a warm environment and killed after 3 h with an excess of pentobarbital sodium injected into the peritoneum. We chose a 3-h interval on the basis of preliminary experience with control rabbits killed 1 min, 3 h,
and 24 h after the tracheal administration of a mixture made by \(^{125}\text{I}\)-SP-A and surfactant labeled in vivo with \[^3\text{H} \text{choline} \text{(Table 1)}\). In those experiments, we found that 3 h after instillation 1) the administered materials were evenly distributed between alveoli and parenchyma, 2) the lung retains enough \(^{125}\text{I}\)-SP-A for counting, and 3) \(^{125}\text{I}\)-SP-A leaves the airways with kinetics similar to those of SP-A labeled in vivo (Table 1) (2). Finally, we chose a 3-h interval to reproduce in vivo the acute setting of exposure to amiodarone studied in vitro.

After exsanguination, the thorax was opened, and a 20-gauge needle was inserted and tied to the proximal trachea for alveolar lavage. The lungs were lavaged sequentially with 4–5 ml of 0.9% normal saline. Each aliquot was infused and withdrawn by syringe three times. The aliquots recovered from individual animals (85–90% of the fluid infused) were pooled and used to count cells, to measure phospholipids, to count \(^{125}\text{I}\) radioactivity (total and TCA precipitable), to count the radioactivity associated with DPPC (25), and to estimate cell viability by trypan blue exclusion. An aliquot of the lavage fluid was centrifuged for 10 min at 500 \(g\), and the resulting supernatant was used to measure proteins, \(\beta\)-hexosaminidase activity, and LDH (2). The washed lungs were homogenized in cold saline and used immediately to count \(^{125}\text{I}\) radioactivity and to isolate and count DPPC. The radioactivity recovered from lavage fluid and lung tissue is expressed as percent of the dose instilled. We found in separate experiments that DMSO in the fluid instilled did not modify, with respect to previous experience (2), the disposal of labeled SP-A and DPPC, composition of the lavage fluid, and viability of the cells from the lavage.

These experiments were approved by the local committee on the handling of laboratory animals.

The studies in vivo were done on 3-day-old rabbits to reduce the use of radioactive materials and to contain the volume of radioactive waste. The studies in vitro were done on alveolar macrophages collected from adult rabbits because large numbers of cells were needed. We acknowledge that developmental differences could have influenced the results. However, this point was not analyzed in the present study.

Statistical analysis. Data are means ± SE. Differences between two groups were analyzed by unpaired \(t\)-test (two tailed). Differences between more than two groups were analyzed by ANOVA and with Fisher’s protected least significance difference as the post hoc test. The level of significance accepted was 5%.

RESULTS

Morphology. After 24 h of culture, control macrophages had a normal appearance (Figs. 1 and 2). On the other hand, macrophages cultured with 10 \(\mu\)M amiodarone presented an increased number of vesicles, sometimes occupying most of the cytoplasm, and an increased surface density of inclusion bodies containing amorphous and/or multilamellar electron-dense structures. The surface density of mitochondria and lysosomes had decreased (Figs. 1–3). The endoplasmic reticulum was not significantly influenced by 24 h of incubation with 10 \(\mu\)M amiodarone (Fig. 3).

Viability. Incubation of alveolar macrophages with 1–20 \(\mu\)M amiodarone for 1 h or with 1–10 \(\mu\)M amiodarone for 24 h had no effect on viability (Fig. 4). Incubation with 50 and 100 \(\mu\)M amiodarone decreased the viability so that after 24 h, only 31 and 2%, respectively, of the macrophages remained viable (Fig. 4).

One hour of incubation with 10 \(\mu\)M amiodarone did not increase the extracellular release of LDH above control values and did not change the cell content of cytochrome-c oxidase (data not shown).

Table 1. Recovery of \(^{125}\text{I}\)-SP-A and \[^3\text{H}\]DPPC administered into the trachea of control 3-day-old rabbits

<table>
<thead>
<tr>
<th>Time</th>
<th>(^{125}\text{I})-SP-A</th>
<th>[^3\text{H}]DPPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAL Homogenate Total lung</td>
<td>BAL Homogenate Total lung</td>
</tr>
<tr>
<td>1 min</td>
<td>68.8 ± 3.5 16.3 ± 1.1 85.3 ± 4.1</td>
<td>48.7 ± 3.0 22.4 ± 1.7 71.1 ± 4.4†</td>
</tr>
<tr>
<td>3 h</td>
<td>31.3 ± 1.9 19.6 ± 1.2 50.9 ± 3.0</td>
<td>32.4 ± 2.3 28.9 ± 2.8 61.4 ± 4.0*</td>
</tr>
<tr>
<td>24 h</td>
<td>4.5 ± 1.3 6.1 ± 1.2 10.6 ± 2.5</td>
<td>18.4 ± 3.2 19.4 ± 2.1 38.6 ± 4.3‡</td>
</tr>
</tbody>
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Values are means ± SE in percent of dose administered; \(n\), no. of animals. \(^{125}\text{I}\)-surfactant protein A (SP-A) mixed with natural surfactant labeled in vivo with \[^3\text{H}\]choline was administered. DPPC, dipalmitoylphosphatidylcholine. Significantly different from recovery of \(^{125}\text{I}\)-SP-A from total lung: *\(P = 0.05\); †\(P < 0.05\); ‡\(P < 0.01\) (by unpaired \(t\)-test).
On the basis of these findings, we have assumed that the effects observed in this paper after incubation of alveolar macrophages with 1–10 μM amiodarone were not due to changes in viability.

**Macrophage phospholipids.** The phospholipid content of alveolar macrophages, expressed as micrograms of phospholipids per microgram of DNA, did not change during the 24-h incubation with 1–10 μM amiodarone (data not shown), in agreement with data from Martin et al. (24). On the other hand, incubation with 50 μM amiodarone for 24 h decreased by 39 ± 9% the cell pool of phospholipids (*P* < 0.05 by ANOVA).

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![Fig. 2. Effect of amiodarone on morphology of alveolar macrophages. Compared with control cells (A and B), a 24-h incubation with 10 μM amiodarone induced the formation of inclusion bodies (C, arrows) containing amorphous (D, arrows) and/or multilamellar (D, arrowheads) structures. Bars, 1 μm.](image)

![Fig. 3. Effect of amiodarone on abundance of organelles. Macrophages were exposed for 24 h to amiodarone and then processed for electron microscopy. The areas covered by mitochondria, lysosomes, inclusion bodies, and endoplasmic reticulum were measured and are expressed as percent of area covered by whole cytoplasm. Values are means ± SE. *P < 0.05 vs. control cells by unpaired t-test.](image)

![Fig. 4. Effect of amiodarone on the viability of alveolar macrophages as estimated by trypan blue exclusion. Values are means ± SE; *n* = 6–10 experiments. ND, not done. *P < 0.01 vs. control cells by ANOVA.](image)
Even if the total content of phospholipids per cell did not change during the 24-h incubation with 10 \( \mu \text{M} \) amiodarone, phospholipid metabolism was deeply affected. In fact, the incorporation of choline into saturated PC increased significantly in the presence of 10 \( \mu \text{M} \) amiodarone, whereas at higher concentrations of amiodarone, choline incorporation decreased, likely reflecting unrecoverable injury (Fig. 5).

Considering that the cell pool of phospholipids did not change during incubation with 10 \( \mu \text{M} \) amiodarone, it appears that amiodarone can increase both the synthesis and degradation of phospholipids. The morphometric data presented in Fig. 3, showing that amiodarone increased the yield of inclusion bodies while decreasing the yield of mitochondria and lysosomes, agree with this interpretation but also indicate that amiodarone affected macrophage phospholipid metabolism in a complex way that cannot be fully elucidated with the present approach. For example, changes in different organelles might take place at different times and at different amiodarone concentrations. It is also worth noting that we did not separate different phospholipid classes and that there could be significant changes in phospholipid classes without changes in total phospholipids. One additional interpretation for the increased incorporation of choline observed at 10 \( \mu \text{M} \) amiodarone might be that increased synthesis is due to a repair process of membrane injury induced by amiodarone, although not to lethal levels. It seems unlikely, however, that the observed change in macrophage phospholipid synthesis has implications for surfactant lipid metabolism.

Degradation of exogenous DPPC by alveolar macrophages. One hour of incubation with 0–100 \( \mu \text{M} \) amiodarone did not change the rate of degradation of exogenous DPPC by alveolar macrophages (Fig. 6).

**Distribution of lysosomal enzymes in the postnuclear supernatant.** In this experiment, we centrifuged the postnuclear supernatant over a gradient of Percoll-sucrose and analyzed the distribution of \( \beta \)-hexosaminidase and arylsulfatase. These enzymes associate with mature lysosomes, which migrate toward the bottom of the gradient, and with early and late endosomes, which stay at the top (2, 26).

As shown in Fig. 7, 1 h of incubation with 10 \( \mu \text{M} \) amiodarone, while not changing the total activity, changed the distribution of \( \beta \)-hexosaminidase during density gradient centrifugation. In fact, the fraction of total activity migrating toward the bottom of the gradient decreased while that staying at the top increased. Similar results were obtained by analyzing the distribution of arylsulfatase (data not shown).

**Release of \( \beta \)-hexosaminidase by alveolar macrophages.** During the 1-h incubation, control macrophages released 0.6 \( \pm \) 0.1% of the cell pool of \( \beta \)-hexosaminidase. The release increased three times in the presence of 10 \( \mu \text{M} \) amiodarone and nine times in the presence of 50 \( \mu \text{M} \) amiodarone (both changes significant; Fig. 8). Because, with 10 \( \mu \text{M} \) amiodarone, the release of LDH was not different from the control value, the observed effect indicates a specific injury response. On the other hand, the release of \( \beta \)-hexosaminidase observed with 50 \( \mu \text{M} \) amiodarone could be a nonspecific event of cell death.

Effects of amiodarone on diphtheria toxin uptake and processing. Ten micromolar amiodarone did not interfere with the ability of diphtheria toxin to block protein synthesis (Fig. 9). Results similar to those reported in Fig. 9 were obtained when macrophages were incu-
bated with 20 and 50 μM amiodarone before being exposed to the diphtheria toxin (data not shown). This result indicates that in the presence of amiodarone, diphtheria toxin was correctly internalized and then transferred from the early endosomes to the cytosol.

Association of SP-A with alveolar macrophages. Incubation with 1–50 μM amiodarone did not change the association of 125I-SP-A with alveolar macrophages (n = 5 experiments; data not shown).

Degradation of SP-A by alveolar macrophages. Amiodarone inhibited the degradation of 125I-SP-A by alveolar macrophages (Fig. 6). The effect was significant at a concentration of 10 μM, and 50% inhibition was reached between 10 and 20 μM. Interestingly, 50 μM amiodarone decreased SP-A degradation to 15 ± 4% of the control value while decreasing viability to 69 ± 5% of the control value.

The effect of amiodarone on the degradation of SP-A by alveolar macrophages was also analyzed in an experiment in which cells pretreated with 0 or 50 μM amiodarone were exposed to 125I-SP-A for 15 min and then the distribution of radioactivity in the postnuclear supernatant was analyzed immediately or after 30 min. As shown in Fig. 10, after a 15-min incubation with 125I-SP-A, there was no difference between control and treated cells in the recovery and distribution of label in the postnuclear supernatant, indicating that amiodarone did not affect the uptake of SP-A. During the chase, however, the cells pretreated with amiodarone retained a greater fraction of incorporated radioactivity, indicating that amiodarone impairs the degradation of SP-A.

In two experiments, we tested also the effect of 1, 2, 5, and 10 μM desethylamiodarone, the main metabolite of amiodarone, on the degradation of SP-A (15). We found that 10 μM desethylamiodarone decreased the degradation of SP-A to 48% of the control value, slightly more than with 10 μM amiodarone, which decreased the degradation to 65 ± 5% of the control value (Fig. 6). However, different from amiodarone (Fig. 4), 10 μM desethylamiodarone decreased macrophage viability to 89% of the control value.

Effect of amiodarone on the clearance of SP-A and DPPC from the airways. The administration of amiodarone into the trachea at the concentrations used in this experiment did not change the concentration of phospholipids, the activity of LDH or β-hexosaminidase

![Graph showing the distribution of β-hexosaminidase among fractions of the postnuclear supernatant.](http://ajplung.physiology.org/)

**Fig. 7.** Effect of amiodarone (amiod) on the distribution of β-hexosaminidase among fractions of the postnuclear supernatant. After a 1-h incubation, cells were homogenized, the postnuclear supernatant was centrifuged over a gradient of Percoll-sucrose, and the distribution of β-hexosaminidase in fractions from the gradient was measured. Values are means ± SE of activity of each fraction expressed as percent of total activity recovered; n = 4 experiments. Significantly different from 0 μM amiodarone: *P < 0.05; **P < 0.02 (by unpaired t-test).
dase, or the number of cells present in lung lavage fluid over 3 h (data not shown). Protein concentration and the viability of the cells from lavage also remained unchanged except for the highest concentration of amiodarone (Fig. 11).

In control rabbits 3 h after administration, we recovered $51 \pm 3\%$ of instilled SP-A and $61 \pm 4\%$ of instilled DPPC from the whole lung (Table 1, Fig. 12). The recovery was greater for DPPC than for SP-A ($P < 0.05$ by unpaired t-test), in agreement with the view that in vivo SP-A is turned over at a faster rate than DPPC (see Table 1, recovery at 24 h, and Ref. 2).

Amiodarone had marked effects on the way the lung handles $^{125}$I-SP-A. In fact, amiodarone did not change the recovery of radioactivity from the airways but increased the recovery from the homogenate in a dose-dependent way (Fig. 12). The change started to be significant when 50 nmol of amiodarone were coadministered with labeled surfactant, that is, at a dose 25 times smaller than that needed to cause lung damage as indicated by protein concentration and cell viability in lavage fluid (Fig. 11). Counts accumulating in the homogenate under the influence of amiodarone represent mostly undegraded SP-A. In fact, in rabbits re-

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**Fig. 10.** Effect of amiodarone on SP-A processing after uptake. Alveolar macrophages were incubated with or without (control) amiodarone for 1 h, exposed to 1 $\mu$g/ml of $^{125}$I-SP-A for 15 min (15'), and then homogenized immediately or after a 30-min (30') chase. The post-nuclear supernatant was then centrifuged over a gradient of Percoll-sucrose, and the distribution of radioactivity was analyzed. cpm, Counts/min. Data are representative of 2 experiments.

**Fig. 11.** Effect of amiodarone administered into the trachea of 3-day-old rabbits on bronchoalveolar lavage (BAL) fluid protein concentration (A) and cell viability as estimated by trypan blue exclusion (B). BAL fluid was collected 3 h after instillation. Values are means ± SE; n = 5 rabbits/dose. *P < 0.01 vs. control rabbits by ANOVA.
receiving the highest dose of amiodarone, the radioactivity of the homogenate that could be precipitated with 20% cold TCA was 98.1 ± 0.1% of total. On the other hand, in control rabbits, the radioactivity of the homogenate that could be precipitated with 20% TCA was 89.9 ± 1.0% of total (P < 0.01 by unpaired t-test). These results indicate that in vivo amiodarone inhibits the degradation of SP-A taken up into the tissue compartments.

In striking contrast to these observations, the clearance of DPPC administered through the trachea was unaffected by the coadministration of amiodarone (Fig. 13).

To summarize, in vivo amiodarone inhibits the degradation of SP-A but not that of DPPC, in agreement with the results obtained with isolated alveolar macrophages.

DISCUSSION

In alveolar macrophages, amiodarone alters the endocytic pathway. This pathway has been operationally delineated by analyzing the distribution of signal proteins, receptors, or enzymatic activities and by following the movement of tracers that follow specific tracks along it (reviewed in Ref. 26). According to the accepted view, endocytosis results in the delivery of internalized molecules to the early endosomes, which are the site where receptor-ligand complexes dissociate and where unoccupied receptors, free ligands, and other molecules internalized with the fluid phase are sorted. Unoccupied receptors and other components to be returned to the plasma membrane collect into recycling vesicles that shuttle toward the plasma membrane either directly or after migrating to the perinuclear cytoplasm.

Materials to be degraded are transferred to the late endosomes and then to the lysosomes. Late endosomes present internal membranes organized as multilayers or as vesicles in a vesicle; have a low density on Percoll gradient centrifugation; are enriched with specific markers like lgp/LAMPs, Rab7, Rab9, syntaxin 7, and the cation-independent mannose 6-phosphate receptor (MPR); and contain active lysosomal hydrolases (26, 28). Lysosomes are distinguished from late endosomes on the basis of their greater density, the lack of MPR, and the property of being the final site of accumulation of internalized macromolecules. Most of newly synthesized lysosomal hydrolases emerge from the trans-Golgi bound to the MPR and in this bound form are transferred to the late endosomes and then, after dissociation from the MPR, to the lysosomes. The late endosomes thus represent a crucial crossroad involved both in the traffic of endocytosed materials and in the biogenesis of the lysosomes.

Amiodarone does not interfere with the ability of macrophages to move molecules from the exterior of the cell to the early endosomes. In fact, in the presence of amiodarone, diphtheria toxin is taken up, processed, and translocated to the cytosol. Because transfer of the toxin to the early endosomes involves clathrin-coated vesicles and because hydrolysis of the toxin in the early endosomes requires a slightly acidic pH (34), it appears that amiodarone does not interfere with formation of clathrin-coated vesicles or with acidification of the early endosomes.

Amiodarone at a concentration that does not affect cell viability inhibits the degradation of SP-A by alveolar macrophages. Because amiodarone does not retard the appearance of SP-A in the postnuclear supernatant and does not interfere with the movement of diphtheria

![Graph](https://example.com/graph.png)
toxin from the exterior of the cell to the early endosomes, we conclude that the defect in the degradation of SP-A is likely to be located at the level of the late endosomes and/or the lysosomes. This interpretation is supported by the fact that amiodarone-treated cells present an increased surface density of vesicles containing multilamellar membranes (the hallmark of the late endosomes), have a decreased surface density of normally sedimenting lysosomes, and display a disturbed traffic of lysosomal enzymes. In the present experiments, the inhibition of SP-A degradation and the increase in the release of β-hexosaminidase were significant at a concentration of 10 μM amiodarone. Because it has been shown that patients treated chronically with oral amiodarone (200–800 mg/day) can achieve plasma concentrations ranging from 0.8 to 7.2 μg/ml (1.2–11.2 μM) (15) and that much greater plasma concentrations can be achieved during intravenous administration of amiodarone (18), we conclude that the present results might be clinically relevant.

**Amiodarone inhibits in vivo the degradation of SP-A.** To study the effect of amiodarone in vivo, we coadministered labeled surfactant together with increasing amounts of amiodarone into the trachea of 3-day-old rabbits and analyzed the distribution of DPPC and SP-A in alveolar and tissue compartments after 3 h. We used this approach because the tracheal administration of amiodarone (1.25 mg administered two times) is a well-established way to induce pulmonary fibrosis in hamsters (6, 8).

We found that amiodarone impairs the disposal of SP-A by inhibiting the degradation of SP-A in the tissue compartment. The decreased degradation of SP-A could explain the recent observation (27), based on histochemistry, that rats chronically receiving amiodarone accumulate SP-A in the alveoli.

As shown in Fig. 12, the minimum amount of amiodarone needed to obtain a significant accumulation of SP-A in the lung was 50 nmol (32.3 μg), a value smaller than that needed to induce fibrosis. This fact and the finding that amiodarone has a direct effect on isolated macrophages suggest that the changes in the turnover of SP-A induced by amiodarone in our animal model represent a direct effect of the drug rather than an effect mediated by inflammation.

**A mechanism of lung damage by amiodarone.** The cationic amphiphilic nature of amiodarone might explain why the terminal organelles of the endocytic pathway are preferentially damaged. Indeed, cationic amphiphilic drugs easily enter the cells and their organelles but, on exposure to an acidic milieu, become insoluble and accumulate to levels that may exceed the extracellular concentration by orders of magnitude (10). Because late endosomes and lysosomes have a strongly acidic interior (26), they are especially prone to accumulate amiodarone. Thus damage to these organelles might impair the ability to degrade exogenous substances like SP-A and direct enzymes normally transported to the lysosomes toward the exterior of the cell. The increased extracellular delivery of lysosomal enzymes might then cause damage and start a cascade of proinflammatory events, but immune or inflammatory mechanisms of lung injury were not explored in this study.

When amiodarone was administered into the trachea of 3-day-old rabbits, we did not find increased levels of β-hexosaminidase in lung lavage fluid. This finding contrasts with the increased release of β-hexosaminidase observed in macrophages incubated with amiodarone. The discrepancy could be explained by the fact that the enzyme-releasing activity of amiodarone could be especially relevant in macrophages (9), to reuptake of the enzyme after secretion (a well-known property of lysosomal hydrolases) (20), or to inactivation of the enzyme after release.

**Effect of amiodarone on lung phospholipids.** In vitro 10 μM amiodarone increased the surface density of inclusion bodies containing multilamellar membranes and increased the incorporation of choline into DPPC, whereas the ability to degrade exogenous DPPC did not change. Because in vitro amiodarone is a strong inhibitor of lysosomal phospholipase A₂ (24), these results suggest that the degradation of exogenous phospholipids by alveolar macrophages may not involve the lysosomal compartment, in agreement with recent findings from our laboratory (2). In vivo amiodarone administered into the trachea over a wide range of concentrations had no effect on the ability to take up and degrade surfactant DPPC. It is therefore tempting to conclude that the intracellular accumulation of membranes induced in macrophages by amiodarone is more likely the result of a derangement in the turnover of cell phospholipids rather than the result of a decreased degradation of surfactant phospholipids. It is worth noting, however, that the tracheal administration may not represent a good model in which to study the effects of amiodarone on the turnover of lung lipids because in some animal models, the oral administration of amiodarone causes accumulation of lung phospholipids, whereas the tracheal administration causes fibrosis (6, 8). Furthermore, the acute effects of amiodarone observed in the present experiment may not reproduce the effects induced by the chronic exposure to the drug.

To summarize, the acute administration of amiodarone inhibits the degradation of SP-A and causes the release of lysosomal enzymes. The alveolar accumulation of SP-A observed in animals with amiodarone-induced lung toxicity could be due to decreased degradation. The release of lysosomal enzymes could contribute to the genesis of lung damage induced by amiodarone.

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