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MECHANISM OF AMINOGLYCOSIDE-INDUCED LYSOSOMAL PHOSPHOLIPIDOSIS: *IN VITRO* AND *IN VIVO* STUDIES WITH GENTAMICIN AND AMIKACIN

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Abstract—Gentamicin, a widely used aminoglycoside antibiotic, is concentrated in lysosomes of proximal tubular cells of the kidney, and induces therein an accumulation of myelin-like material. We show that treatment of rats with Gentamicin (10 mg/kg, 7 days) induces a loss of activity of lysosomal sphingomyelinase and phospholipase A_1, associated with an increase in the amount of total lipid phosphorus in the kidney cortex. *In vitro*, Gentamicin is shown by gel permeation to bind to phospholipid bilayers (liposomes) under conditions which mimic the lysosomal environment (acid pH and presence of phosphatidylinositol). The reversal of this binding by an increase in the ionic strength (> 0.04) suggests electrostatic interaction between the hydrophilic, polycationic aminoglycoside and the negatively charged phospholipids. Binding of Gentamicin impairs the hydrolysis of phosphatidylcholine present in the bilayer, by lysosomal phospholipases A_1 and A_2 from the liver or kidney. We also show that lysosomal sphingomyelinase is readily and irreversibly inactivated by liposomes in the absence of detergent.

The lysosomal phospholipidosis induced by Gentamicin in the kidney, as in cultured cells [Aubert-Tulkens *et al.*, *Lab. Invest.* **40**, 481 (1979)] appears therefore to be a direct consequence of the lysosomotropic character of this drug and its ability to inhibit therein phospholipid breakdown. Amikacin, a semi-synthetic aminoglycoside, binds more loosely to phospholipid bilayers, induces less inhibition of phospholipases *in vitro* and is less taken up by tubular cells *in vivo*. Accordingly, Amikacin does not provoke significant lysosomal phospholipidosis or loss of sphingomyelinase and phospholipase A_1 activities *in vivo* at the doses and time investigated (0–40 mg/kg, 7 days). Inasmuch as Amikacin is reported to be less toxic to the kidney, we suggest that lysosomal alterations are an early and significant step in aminoglycoside-induced nephrotoxicity.

Aminoglycoside antibiotics are essential and widely used in the control of severe Gram (−) sepses. Their use is, however, associated with oto- and nephrotoxicity [1]. Clinically detectable alteration of kidney function occurs in 10–25% of the patients treated with Gentamicin, the most widely used aminoglycoside [2, 3], with probably a higher incidence in aged or critically-ill patients [4].

The earliest and most conspicuous alteration of kidney ultrastructure is the deposition of lamellar material, with a periodicity of about 5 nm, in the lysosomes of proximal tubular cells, suggesting the accumulation of polar lipids [5, 6]. These alterations are seen to various extents in animals treated with low doses of most aminoglycosides. They have also been reported in humans after a few days of treatment [7, 8]. It is thought—but no proven—that lysosomal disturbance and overloading is a cause of further cell damage leading to tubular necrosis, a characteristic pathological feature of aminoglycoside-induced nephrotoxicity in animals and man [5, 6, 9–11]. This interpretation has been rationalized by the finding that Gentamicin accumulates in proximal tubular cells where it is localized primarily in the lysosomes after pinocytic uptake from the luminal fluid [12–14].

Using a model of cultured cells, we found that several aminoglycosides can accumulate in lysosomes [15]. Gentamicin was shown [16] to induce in these cells an accumulation of phospholipids and a loss of activity of the lysosomal sphingomyelinase and phospholipase A_1 (data on the latter enzyme are unpublished). These biochemical alterations were associated with the accumulation of lamellar material within the lysosomes resembling greatly that observed in the kidney. These findings have suggested to us that aminoglycoside could induce a similar impairment of phospholipid breakdown within the lysosomes of the kidney, which would explain their ultrastructural alteration. We report here on biochemical studies of the effect of Gentamicin on the renal cortex from rats treated with low doses of this antibiotic. We also report on *in vitro* experiments undertaken to uncover the molecular mechanism of this drug-induced phospholipidosis. Throughout our work we have compared Gentamicin with another aminoglycoside used in clinical practice, Amikacin, since the latter has been shown to be less toxic both *in vivo* (histopathological alterations) [17] and in cultured cells (lysosomal phospholipidosis)

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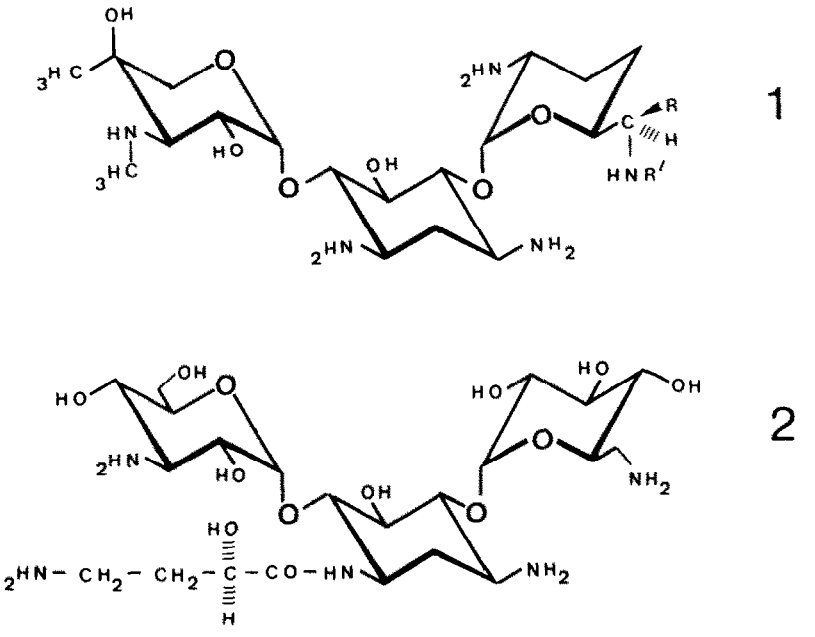

Fig. 1. Structural formulae of Gentamicin (1 [19]) and Amikacin (2 [20]). Commercially available Gentamicin is a mixture of three major subcomponents (C_1: R = R' = CH_3; C_{1a}: R = R' = H; C_2: R = CH_3, R' = H; mass ratio approximately 30:30:40). The systematic name of Gentamicin C_{1a} is: O-3-deoxy-3(methylamino)-4-*C*-methyl-β-L-arabinopyranosyl-(1→6)-O-[2,6-diamino-2,3,4,6-tetradeoxy-α-D-erythro hexopyranosyl-(1→4)]-2-deoxy-D-streptamine; the systematic name of Amikacin is: O-3-deoxy-3-amino-α-D-glucopyranosyl-(1→6)-O-[6-deoxy-6-amino-α-D-glucopyranosyl-(1→4)]-N^1-(L-4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine.

[18]. The structural formulae [19, 20] and systematic names of both compounds are given in Fig. 1.

MATERIALS AND METHODS

Animal studies. Female Wistar and Sprague–Dawley rats (200–250 g) were used throughout the experiments. The aminoglycosides dissolved in 9‰ NaCl were injected intraperitoneally (in approximately 0.5 ml) once a day for 7 days. Control animals receiving 0.5 ml of 9‰ NaCl were included in each study. The animals were killed by decapitation 24 hr after the last injection. The cortex was dissected from each kidney and homogenized in 49 volumes of distilled water using a conical tissue grinder fitted with a ground glass pestle. The protein concentration in the homogenates was measured as described by Lowry *et al.* [21]. Total phospholipids were extracted as described by Bligh and Dyer [22] except that the aqueous phase was made acidic with 0.1 N HCl. After mineralization, phosphorus was assayed as described by Bartlett [23]. Sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12) activity was measured with [*N*-methyl-^{14}C]sphingomyelin at pH 5.5, in the presence of 0.1% taurocholate and 0.1% Triton X-100 [24]. Addition of Ca^{2+} or EDTA had no influence on the activity of this enzyme, which is considered to be lysosomal in several tissues [25]. Phospholipase A_1 (phospholipid 1-deacylase, EC 3.1.1.32) was assayed with L-α-phosphatidylcholine di-[1-^{14}C]palmitoyl, in the presence of 0.25% taurocholate and 0.25% Triton X-100 [26]. Under these conditions, only phospholipase A_1 is active (checked with 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine and phosphatidylcholine [*N*-methyl-^{14}C]dipalmitoyl). The activity was maximal at pH 4.0 and was not affected by EDTA (20 mM); Ca^{2+} and Mg^{2+} (20 mM each) were slightly inhibitory (approximately 12%). This activity is thus similar to that found in the soluble fraction from purified lysosomes from the liver [27, 28]. Antibiotic content was measured by a microbiological assay of the homogenates, as described previously [15].

Preparation of liposomes. Liposomes (mixture of multi- and monolamellar) were prepared essentially as described by one of us previously [29], except that sonication was performed with a Branson B12 sonifier equipped with a 3-mm diameter titanium probe. We used the following compositions; for binding studies: cholesterol–egg yolk phosphatidylcholine–wheat-germ phosphatidylinositol (molar ratio 5.5:8:3); for enzymatic studies half the phosphatidylcholine was replaced by bovine brain sphingomyelin. Liposomes, stored at 4° under nitrogen, were used within a week.

Binding studies. For a typical experiment approximately 50 μg of aminoglycoside (free base) and 8 mg of liposomes (lipids) were incubated for 1 hr at 37° in 1 ml, and this preparation was then applied to a 1.6 × 24 cm column packed with Sepharose 4B. Elution was made at a rate of 0.6 ml/min and 2-ml fractions were collected. Aminoglycosides were

assayed by fluorimetry after reaction with fluorescamine [30]. Samples containing up to 1.5 μg of aminoglycosides were mixed with 1.2 ml of 0.5 M borate buffer, 1.25% Triton X-100, pH 9, in a final volume of 1.5 ml. 0.5 ml of 0.1 mg/ml fluorescamine in acetone was added to each sample and mixed thoroughly. Fluorescence was read at a wavelength of 475 nm, with excitation at a wavelength of 399 nm.

Enzymatic assays on liposomes. Degradation of phospholipids in liposomes was studied using the soluble fraction of isolated liver lysosomes (isolated from rats treated with Triton WR-1339, as described by Trouet [31]), or homogenates of the kidney cortex. Phospholipase activities were assayed with 0.1 mg of liposomes and 5.2 μg of lysosomal protein, or 0.15 mg of liposomes and 50 μg of kidney homogenate protein. In each case, liposomes contained labeled phosphatidylcholine (1-palmitoyl, 2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine; 0.14 μCi/μmole).

Incubation was carried out at 37° in 20 μl of 4 mM acetate buffer, pH 5.4 (preliminary experiments showed this pH to be the optimum). The reaction was stopped by the addition of 50 μl of methanol. The mixture was dried at 37° under a gentle nitrogen stream. The residue was dissolved in 15 μl of chloroform–methanol [1:1 (v/v)] and spotted on a precoated thin-layer silica gel plate (Merck, Darmstadt, F.R.G.). Ascending chromatography was performed with chloroform–methanol–acetic acid–water (25:15:18:4). Plates were cut in strips after the phospholipids had been visualized by spraying bromophenol blue. Radioactivity was measured by scintillation counting. For phosphorus determination, the gel was scraped off from the plate and mineralization was performed in 60% perchloric acid for 90 min at 210°, after which the Bartlett procedure was used. Recovery of radioactivity and phosphorus was between 95 and 110%.

For sphingomyelinase activity, liposomes contained 0.14 μCi of [*N*-methyl-^{14}C]sphingomyelin per μmole of sphingomyelin. Release of [*N*-methyl-^{14}C]phosphorylcholine was measured as TCA-soluble radioactivity [24].

Materials. Animals were obtained from Charles River (France). Gentamicin (mixture of C_1, C_{1a} and C_2 subcomponents) and Amikacin were supplied by Schering Corp. (Kenilworth, NJ) and Bristol Myers International Corp. (New York, NY). Animal studies were performed with the sulfate salt preparations used in clinical practice. For the inhibition studies *in vitro*, we used samples of purified antibiotics (sulfate salts, generously given by the manufacturers), and the pH was adjusted to 5.4 prior to use. For the binding studies we used the free base prepared from the sulfate by titration with barium hydroxide. The contents of the final preparations were checked by microbiological assay, and all concentrations are expressed in equivalents of free base.

Natural glycerophospholipids were obtained from Lipid Products (Redhill, U.K.; we used grade 1 products). Synthetic glycerophospholipids and bovine sphingomyelin were purchased from Sigma Chemical Co. (St. Louis, MO). Radioactive phospholipids were from the Radiochemical Center (Amersham, U.K.). The purity of labelled and non-labelled lipids was checked by TLC, and found to be higher than 98–99%, in accordance with suppliers' specifications.

RESULTS

Animal studies

Figure 2A shows the influence of treatment with either Gentamicin or Amikacin on the activities of sphingomyelinase and phospholipase A_1 from the kidney cortex of Sprague–Dawley rats.

The doses of antibiotics used are close to those recommended in clinical practice for Gentamicin (3–5 mg/kg) and Amikacin (15 mg/kg) [1]. The four-fold higher dosage of Amikacin did not result in a commensurate higher uptake by the kidney cortex. Indeed, after 7 days of treatment with 10 mg/kg Gentamicin or 40 mg/kg Amikacin, tissue levels were 1.4 ± 0.2 and 1.9 ± 0.2 μg/mg of protein (N = 8) for the total cortex. Gentamicin induces a significant decrease in the activity of both enzymes, whereas Amikacin has almost no effect at the doses used. To check on the specificity of this effect, several lysosomal hydrolases that do not act on phospholipids were assayed and showed normal activity. The activities of cytochrome oxidase, glucose-6-phosphatase and phosphorylase were not affected either.

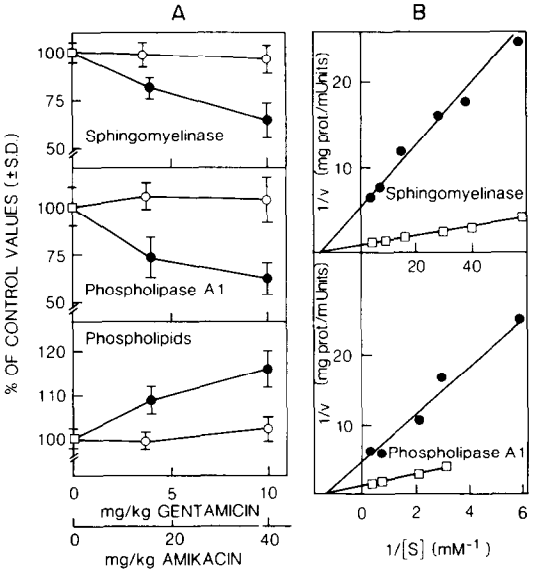

Fig. 2. Influence of treatment with Gentamicin (●) or Amikacin (○) on the activities of sphingomyelinase and phospholipase A_1, and on the total phospholipid content in the rat kidney cortex. Panel A: Sprague–Dawley rats were treated for 7 days with one daily injection of the antibiotic at the dose shown on the abcissa. The activity of the enzymes and the lipid phosphorus content (± S.D., N = 5) are expressed as percentages of the values found for control animals (injected with 0.9% NaCl). Panel B: Wistar rats were treated with 100 mg/kg of Gentamicin for 9 days (●), or with 0.9% NaCl (□). Activities of sphingomyelinase and phospholipase A_1 were measured at increasing substrate concentrations. Results are presented as a double-reciprocal plot of substrate concentration and reaction velocity.

The lower panel of Fig. 2A shows that Gentamicin treatment results in a significant increase in the content of total lipid phosphorus in the kidney cortex. TLC of the whole cortex extract (not illustrated) showed a non-specific increase in the concentration of all major phospholipids (phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin), and no indication of a selective, marked increase in minor phospholipids. Electron microscopic examination of Gentamicin-treated animals showed an abundance of concentric lamellar material in the lysosomes of proximal tubular cells, as was reported earlier [5]. These alterations were much less marked in Amikacin-treated animals. In either case, other cytoplasmic organelles were unaffected. Detailed studies on the time and dose dependence of the treatment on the enzyme activities and lipid phosphorus content, and on the morphometry of lysosomal alterations will be published elsewhere (Carlier *et al.*, manuscript in preparation).

Figure 2B shows the results of kinetic studies on sphingomyelinase and phospholipase A_1 from kidney cortices of Wistar rats treated with a high dose of Gentamicin (100 mg/kg for 9 days). This treatment induces a very marked decrease in the activities of both enzymes. The residual activities show K_m values similar to those of controls. Activity could not be restored by dilution, addition of detergent or freeze-thawing of the preparations. The loss of activity of sphingomyelinase and phospholipase A_1 was only observed in the kidney of *treated* animals. Addition of Gentamicin in the reaction mixture or pre-exposure of the enzyme preparation to Gentamicin at pH 5 and for several hours had no effect.

Binding of Gentamicin to liposomes

In the next step, we studied the interaction between aminoglycosides and phospholipids. To mimic the conditions prevailing *in vivo*, phospholipids were prepared in the form of bilayers (liposomes), which allows us to avoid detergents.

Figure 3A shows a typical elution pattern of the liposome preparation on a Sepharose 4B column in 4 mM acetate buffer, pH 5.4. The first narrow peak (16–22 ml) corresponds to the multilamellar vesicles which are eluted in the void volume of the column. The shoulder is due to the presence of small unilamellar vesicles which are fractionated by the gel permeation. The poor separation of multi- and unilamellar vesicles was frequently observed when the elution was performed at low ionic strength and acid pH. Increasing the ionic strength or pH gave a more marked separation. The elution pattern of Gentamicin on Sepharose 4B is also shown in Fig. 3A. At low ionic strength, no drug was recovered, even when a volume equivalent to the bed volume of the column flowed through. When the eluant was changed to a 0.15 M NaCl–4 mM acetate buffer, Gentamicin was then recovered from 45–55 ml of eluant, consistent with a mean mol. wt of approximately 460.

In the experiment shown in Fig. 3B, Gentamicin (0.1 μmoles) and liposomes (10.6 μmoles of phospholipids) were mixed and incubated at 37° for 1 hr before being applied to the column. About 95% of the Gentamicin is recovered in a peak appearing at the same position as the peak for liposomes. The small amount of Gentamicin retained by the gel was eluted with 0.05 M cacodylate buffer–0.15 M NaCl, pH 7.4, at the same position as free Gentamicin (see panel A). The elution of a mixture of Amikacin (0.1 μmoles) and liposomes is shown in Fig. 3C. Only 46% of Amikacin is associated with the liposome peak whereas the remaining drug was eluted after increasing the pH and the ionic strength. The shape of the elution profile of liposomes in the presence of aminoglycoside showed slight variations from one experiment to another. It remained, however, consistently and clearly separated from the elution profile of free aminoglycoside; the amount of drug bound to liposomes could therefore be accurately determined in all experiments.

The influence of the ionic strength and pH on the binding of Gentamicin to liposomes is shown in Fig. 4. In these experiments, the drug and liposomes were incubated as in Fig. 3, but elution was made with buffers of different ionic strength and/or pH. At pH 5.4, the binding is unaffected by the ionic strength up to a value of 0.044 (0.04 M NaCl–4 mM acetate buffer). At pH 7.4, dissociation is achieved much more readily. At pH 8.4, only half of the Gentamicin was bound, even at low ionic strength. Thus, binding of aminoglycosides to liposomes is reversible, i.e. complexes such as that shown in Fig. 3B can readily be dissociated by exposure to a high ionic strength and/or neutral or alkaline pH.

In vitro *enzymatic studies*

Degradation of phosphatidylcholine. When liposomes that contain 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine are incubated in the presence of a soluble fraction of liver lysosomes, a rapid release of lysophosphatidylcholine is observed (Fig. 5A). The per cent of radioactivity associated with lysophosphatidylcholine is, however, consistently lower than the per cent of phosphorus. In contrast, the specific radioactivity of phosphatidylcholine remains constant throughout. Thus both phospholipases A_1 and A_2 (phospholipid 1-deacylase, EC 3.1.1.32, and phospholipid 2-deacylase, EC 3.1.1.4) are active under our experimental conditions. The amounts of 2-oleoyl-*sn*-glycero-3-phosphocholine (β-lysophosphatidylcholine) and 1-palmitoyl-*sn*-glycero-3-phosphocholine (α-lysophosphatidylcholine) were calculated from the differences between the phosphorus and radioactivity measurements, and these data are shown in Fig. 5B. Liberation of β-lysophosphatidylcholine was much more rapid than that of α-lysophosphatidylcholine which shows that initial degradation is predominantly carried out by phospholipase A_1. Fig. 5A also shows the liberation of radiolabelled oleic acid. Positive identification of this compound was made by TLC with heptane–isopropyl ether–acetic acid (60:40:4). No radioactive 1-palmitoyl, 2-oleoyl glycerol (diglyceride) or 2-oleoyl glycerol (monoglyceride) was observed. Thus no phospholipase C activity (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.-) [32] was detected under our conditions. The release of oleic acid exceeds that of α-lysophosphatidylcholine (compare curve △ in panel A with curve ◐ in panel B). Part of the oleic acid must therefore arise from

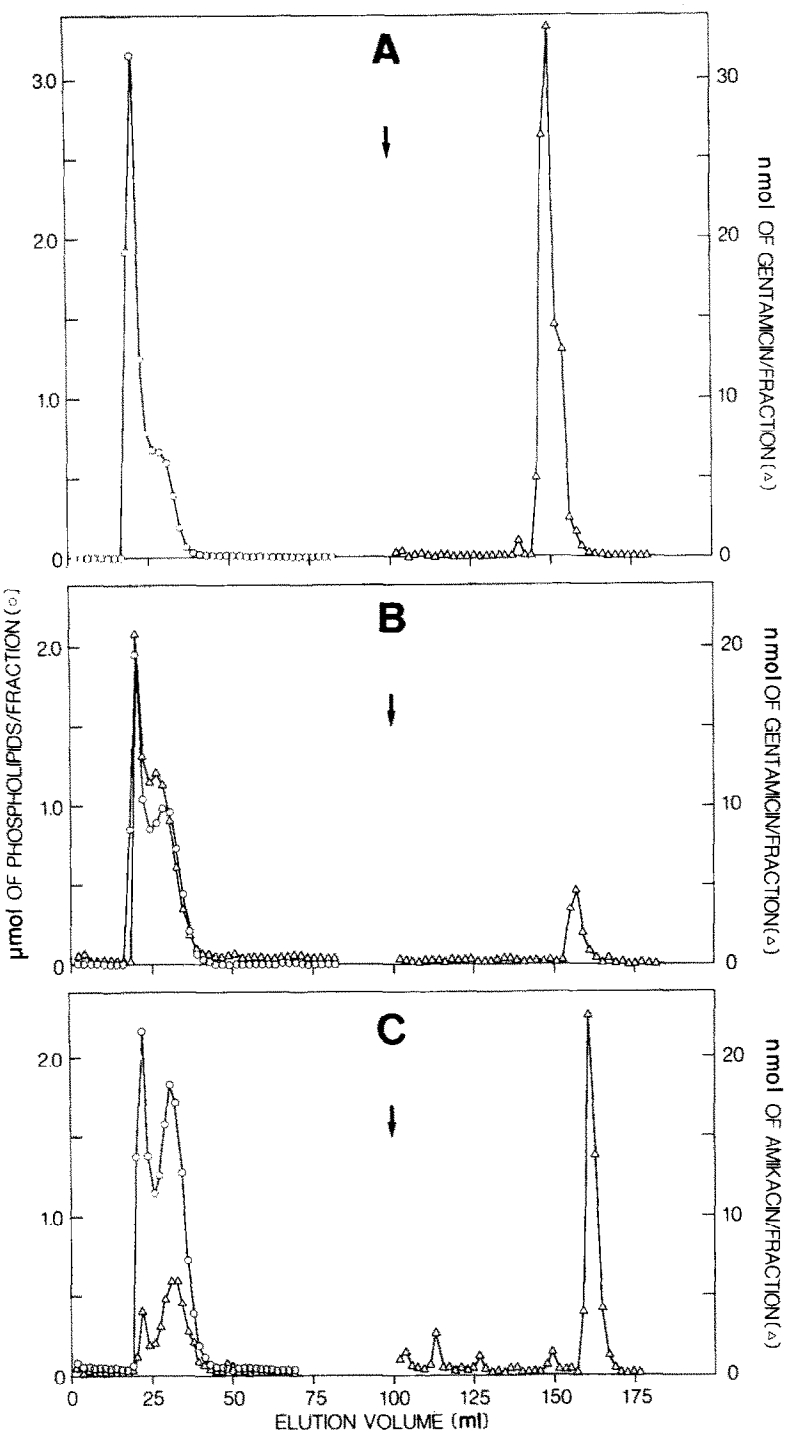

Fig. 3. Elution profiles of aminoglycoside (△) and liposomes (○) after gel permeation on Sepharose 4B. No symbols are shown when readings were not different from those of blanks. Panel A: Elution of 8 mg of liposomes [cholesterol–phosphatidyl choline–phosphatidylinositol (molar ratio 5.5:8:3)] or of Gentamicin (0.1 μmoles, free base) with 4 mM acetate buffer, pH 5.4. At the point indicated by the arrow, the ionic strength of the buffer was increased by addition of 0.15 M NaCl. Panel B: Liposomes (8 mg) were mixed with Gentamicin (0.1 μmoles, free base) and incubated for 1 hr at 37°, before being passed through the column and eluted with 4 mM acetate buffer, pH 5.4. At the point indicated by the arrow, the eluant was changed to 50 mM cacodylate buffer–0.15 M NaCl, pH 7.4. Panel C: Liposomes (9 mg) were mixed with Amikacin (0.1 μmoles, free base) and treated as in the experiment shown in panel B.

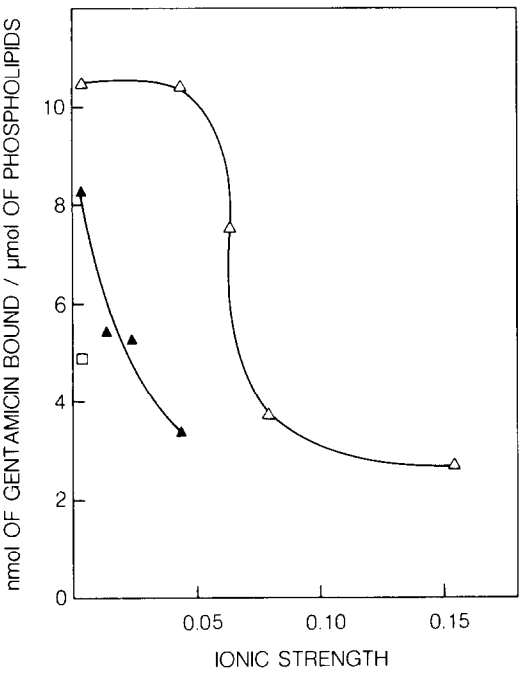

Fig. 4. Influence of the ionic strength and pH of the eluant buffer on the association of Gentamicin to liposomes. The amount of bound Gentamicin was determined by gel permeation as in Fig. 3. Recoveries of the aminoglycoside and phosphorus, in these experiments and those of Fig. 3, were between 88 and 135%, and 85 and 115%, respectively. (△) acetate buffer, pH 5.4; (▲) cacodylate buffer pH 7.4; (□) borate buffer, pH 8.4. All buffers were 4 mM sodium salt; increase in ionic strength was brought about by the addition of NaCl.

another reaction than that catalyzed by phospholipase A_2. We suggest that it is liberated from β-lysophosphatidylcholine (the product of phospholipase A_1), by a lysophospholipase (lysolecithin acyl hydrolase, EC. 3.1.1.-). This release is shown in Fig. 5B. The levelling off of the concentration of lysophosphatidylcholine could, therefore, result from production being matched by degradation. Our approach does not allow us to study directly the degradation of α-lysophosphatidylcholine. In the presence of detergents (0.25% Triton X-100–0.25% taurocholate) no release of fatty acid is observed (inhibition of phospholipase A_2 and/or β-lysophospholipase).

Similar studies were performed with kidney cortex homogenates. Because of much lower specific activities and the presence of endogenous phospholipids, only measurements of radioactivity could be accurately obtained. The pattern of reactions was, however, similar.

The effect of aminoglycosides on the hydrolysis of phosphatidylcholine was then studied. Gentamicin or Amikacin [sulfate salts (Gentamicin·2.5H_2SO_4) and Amikacin·2H_2SO_4) adjusted to pH 5.4] were mixed with liposomes in increasing amounts (up to approximately 0.8 µmoles per 10 µmoles of phospholipids) and incubated for 1 hr at pH 5.4 in 4 mM acetate buffer, after which an equal volume of enzyme preparation was added. Figure 6 shows the effect of aminoglycosides on the hydrolysis of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine. Both antibiotics inhibit almost completely the release of β-lysophosphatidylcholine and oleic acid. This shows that Gentamicin inhibits both phospholipase A_1 and A_2. Figure 5 shows also that Amikacin is a significantly less potent inhibitor of the hydrolysis of phosphatidylcholine. Very similar results were obtained when using the antibiotic preparations made for clinical practice. When the experiments were repeated in the presence of detergents (0.25% Triton X-100–0.25% taurocholate) no inhibition of phospholipase A_1 was observed. Activity of phospholipase A_2 or β-lysophospholipase could not be studied in the presence of these detergents (see earlier).

In another set of experiments we used whole cortex homogenates (53 µg of protein) as the enzyme source, and measured the liberation of radiolabelled lysophosphatidylcholine (activity of phospholipase A_1). The concentrations of antibiotic causing a 50% inhibition (IC_{50}) were 40.7 ± 2.1 and 68.5 ± 1.32 µg/ml (N = 3) for Gentamicin and Amikacin, respectively. In all conditions, sulfate alone (Na_2SO_4, up to 1.6 µmoles per 10 µmoles of phospholipids) had no effect.

Degradation of sphingomyelin. When liposomes prepared with [*N*-methyl-^{14}C]sphingomyelin (0.14 µCi/µmole of sphingomyelin) were incubated with either soluble extracts of liver lysosomes or kidney cortex homogenates, almost no release of labeled phosphorylcholine was observed after up to 24 hr of incubation. Upon addition of 0.1% Triton X-100 and 0.1% taurocholate before, or at the time as, the enzyme addition, activity was, however, observed of a similar magnitude to that measured with the assay method used for *in vivo* studies. If the addition of detergent followed the addition of enzyme, less activity was recovered. Figure 6 shows that 75% of the activity is lost after 1 hr of contact between enzyme and liposomes. No further activity was disclosed upon the addition of a fresh preparation of liposomes, or upon addition of labeled sphingomyelin dispersed with detergents. We also checked by TLC that labeled and unlabeled sphingomyelin was not appreciably degraded during the incubation without detergent. Since lysophosphatidylcholine was produced during this incubation (see Fig. 4), we checked the influence of this compound on sphingomyelinase activity, measured in the presence of detergents, but no effect was noted.

DISCUSSION

The biochemical alterations induced in kidney cortex by Gentamicin amounts close to therapeutic doses are essentially similar to those reported by us earlier for fibroblasts cultivated in the presence of this antibiotic [16]. Moreover, accumulation of Gentamicin was reported in the lysosomes of both cell types [12, 13, 15] and the intracellular concentrations which elicit the lysosomal alterations are within the same range (1–10 µg/mg of protein). The actual intracellular concentration of Gentamicin in proximal tubular cells is probably higher than the values reported for total cortex, since distal tubules

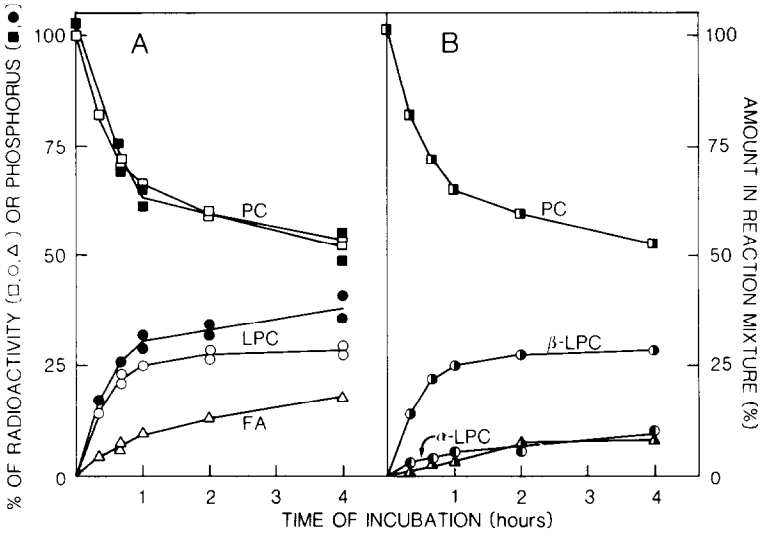

Fig. 5. Hydrolysis of phosphatidylcholine in liposomes by the soluble fraction of liver lysosomes. Liposomes contained 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (0.14 μCi/μmole phosphatidylcholine). All values are expressed as percentages of total radioactivity or phosphorus associated with phosphatidylcholine introduced in the assay mixture. Panel A: PC: phosphatidylcholine, measured by radioactivity (□) or phosphorus (■); LPC: lysophosphatidylcholine, measured by radioactivity (○) or phosphorus (●); FA: oleic acid measured by radioactivity (△). Panel B: Data calculated from panel A. PC (◨): phosphatidylcholine, mean of the data from radioactivity and phosphorus measurement; β-LPC (◑): 2-acyl-*sn*-glycero-3-phosphocholine (β-lysophosphatidylcholine) estimated by measurement of radioactivity; α-LPC (◐): 1-acyl-*sn*-glycero-3-phosphocholine (α-lysophosphatidylcholine) estimated from the difference between lysophosphatidylcholine [phosphorus assay (left panel: ●)] and 2-acyl-*sn*-glycero-3-phosphocholine [radioactivity (left panel: ○)]; oleic acid (▲) released from 2-acyl-*sn*-glycero-3-phosphocholine (β-lysophosphatidylcholine), estimated from the difference between total oleic acid release (△ in left panel) and release of α-lysophosphatidylcholine (◐ in right panel).

and glomeruli do not accumulate much of the drug [12–14].

The selective action of Gentamicin towards the lysosomes of proximal tubular cells *in vivo* results from adsorptive endocytosis of the antibiotic from the luminal fluid after parenteral administration [12–14]. Actually, we could find *in vivo* typical myelin-like figures in the lysosomes of other cells when these are exposed to large amounts of aminoglycosides, namely in conjunctival fibroblasts of animals and patients after subconjunctival injections of Gentamicin [33].

Accumulation of glycerophospholipids (phosphoglycerides) and sphingomyelin in lysosomes results from the loss of activity of phospholipase A_1 and sphingomyelinase. In liver lysosomes, the degradative pathway of glycerophospholipids involves deacylation by phospholipases A [34]. This work shows that phospholipase A_1 is the most active phospholipase towards phosphatidylcholine (Fig. 5), as also reported recently by others (27). The same conclusion seems to apply for the kidney cortex. Moreover, phospholipase A_2 is inhibited by Gentamicin *in vitro* (Fig. 6). On the other hand, phospholipase C [32, 35] does not seem to act appreciably on 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, as also observed by others [27]. On the other hand, sphingomyelinase is responsible for the first degradative step of sphingomyelin in several tissues, both *in vitro* and *in vivo* [25, 36–38].

We have no clear explanation as to the mechanism responsible for the loss of total activity of phospholipase A_1 *in vivo*. Non-specific action of Gentamicin on enzyme synthesis, transport or turn-over are unlikely, since several other enzymes from lysosomes are unaffected. Moreover, no other accumulation apart from that of phospholipids is evidenced. A direct, specific effect of Gentamicin on lysosomal phospholipase A_1 is not likely either, since no influence of the antibiotic is observed *in vitro* on phosphatidylcholine breakdown *in the presence of detergents*. Preexposure of a lysosomal preparation to the antibiotic is also without effect.

Aminoglycosides are polyaminated, hydrophilic molecules. Several reports point to a binding of these antibiotics to negatively-charged phospholipids [39–42]. Our studies demonstrate that such a binding occurs, under conditions that are relevant to those prevailing in lysosomes, i.e. a large amount of phosphatidylinositol [43, 44] and an acid pH [45]. Reversibility of binding upon an increase in pH and ionic strength suggests that it is mainly mediated by electrostatic forces. The effect of pH is particularly interesting since it could contribute to the selective action of the drug towards lysosomes.

Under conditions in which binding to liposomes is demonstrated, Gentamicin markedly inhibits the degradation of phosphatidylcholine included in the bilayer. This inhibition is observed with Gentamicin concentrations equal or below those observed in

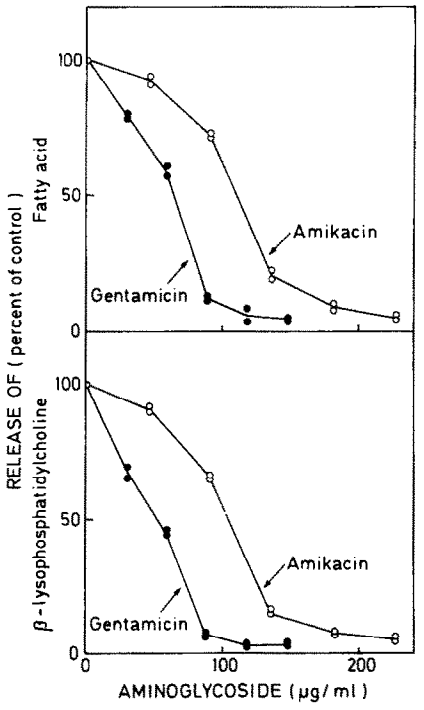

Fig. 6. Effect of aminoglycosides on the hydrolysis of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine in liposomes. Liposomes (10 μmoles of phospholipids) were mixed with Gentamicin (●) or Amikacin (○), both the sulfate salt adjusted to pH 5.4, and pre-incubated at 37° for 1 hr in 4 mM acetate buffer, pH 5.4. The reaction was started by the addition of an equal volume of the soluble fraction of liver lysosomes (approximately 5 μg of protein) and incubation was carried out at 37° for a further 30 min. The actual concentrations of antibiotic in the final assay mixture are shown on the abscissa. The upper part of the diagram shows the release of labeled oleic acid (activities of phospholipase A_2 and β-lysophospholipase); the lower part the release of labeled lysophosphatidylcholine (2-oleoyl-*sn*-glycero-3-phosphocholine) (activity of phospholipase A_1). The figure shows the results of duplicate determinations. All values are percentages of the amount released in the absence of antibiotic (fatty acid 5.8 ± 1; lysophosphatidylcholine: 16.1 ± 4.1; both per cent of total radioactivity; N = 6).

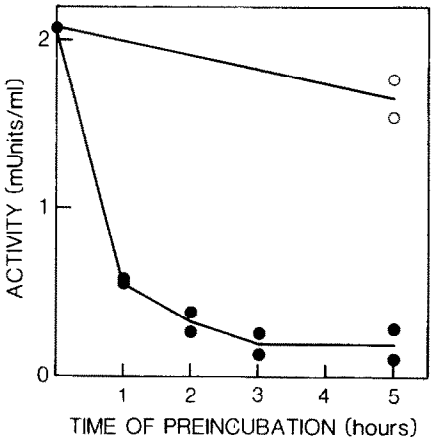

Fig. 7. Effect of the pre-incubation of a soluble lysosomal fraction (1 μg of protein) in the presence of liposomes (●) on sphingomyelinase activity. Liposomes contained 0.14 μCi of [*N*-methyl-^{14}C]sphingomyelin per μmole of sphingomyelin. Pre-incubation was carried out in the absence of detergents and the reaction was initiated by addition of 0.1% Triton X-100 and 0.1% taurocholate. In control experiments (○) the enzyme preparation was incubated in buffer alone, and liposomes were added simultaneously with detergents.

lysosomes (see discussion in Refs. 14–16). The present study has not distinguished between competitive and non-competitive inhibition, and no information is available as whether Gentamicin acts on the substrate alone or on an enzyme–substrate complex. Should the latter occur *in vivo*, it could explain the irreversible loss of activity of phospholipase A_1 (Fig. 2).

The loss of activity of sphingomyelinase observed in the kidney cortex of Gentamicin-treated rats could be a secondary effect of phospholipid accumulation. Our *in vitro* data show that this enzyme is quickly and irreversibly inactivated in the presence of phospholipid bilayers. This may occur very early during Gentamicin treatment, i.e. when only a modest accumulation of phospholipids has taken place. Our earlier report [16] that sphingomyelinase deficiency seems to precede phospholipid overloading is therefore not contradictory to the present hypothesis. Moreover, inactivation of sphingomyelinase will contribute to a further development of lysosomal phospholipidosis.

The experiments reported in this and earlier publications [14–16] provide therefore a rational explanation for the lysosomal alterations induced *in vivo* by aminoglycosides and which appear a direct consequence of the intrinsic lysosomotropic character of these drugs [46] and of their ability to inhibit the breakdown of phospholipids. Particularly noteworthy is the fact that Amikacin displays a lower affinity for the phospholipid bilayer and is taken up less by renal cortex tissue. The result should be a lesser effect on the lysosomes *in vivo* despite a higher dosage, as is indeed shown in Fig. 2. Direct correlation between the enzymatic studies *in vitro* (Fig. 6) and those performed with tissues from treated animals can only be made with great caution, since the *in vitro* experiments were done under conditions that maximize the binding of aminoglycosides to liposomes. In particular, the ionic strength was kept low and the inhibition of phospholipase activity *in vivo* may require higher aminoglycoside concentrations. Moreover, Amikacin has less ionizable amines than Gentamicin (Fig. 1) and should be more easily displaced from phospholipid bilayers by cations, as found recently in studies on competition between Ca^{2+} and aminoglycosides towards phosphatidylserine monolayers [42].

Our studies do not provide an explanation regarding the mechanism by which aminoglycosides induce cell death, as observed with cultured cells or in kidney tubules *in vivo*, after exposure to these drugs in large amounts or for prolonged times [5, 10, 16, 17]. Sphingomyelinase deficiency or phospholipid accumulation *per se* do not seem critical. On one hand, cells from patients with a complete lack of sphingomyelinase (Niemann–Pick disease

type A or B) can be maintained in culture [37]. On the other hand, cells exposed to *amphiphilic* drugs or tissues of animals treated with these compounds accumulate very large amounts of phospholipids, without early cell death [56].

It has been reported that lysosomes accumulate acidic phospholipids such as bis [monoacylglyceryl]phosphate in various conditions including the Niemann–Pick-disease [48], drug-induced phospholipidosis [47, 49, 50] or mere overloading of lysosomes by undigestible substances [51]. This accumulation is, however, not associated with early cell death. Although our chromatographic analysis, performed on the whole cortex, does not exclude a mild accumulation of acidic phospholipids after Gentamicin treatment, this is therefore unlikely to be the cause of tubular necroses. Rather the key factor could be the ability of Gentamicin to inhibit lysosomal phospholipases A, which may be essential enzymes to cells, e.g. for membrane turn-over and fusion [28] or for clearing toxic phospholipids. It is intriguing that no inborn deficiency of phospholipases A has ever been uncovered. Whatever the exact mechanism, it should be stressed that Amikacin induces less histopathological alteration, including necrosis in proximal tubules as compared to Gentamicin [17]. Moreover, derivatives of Gentamicin, either more or less toxic than the parent drug, have recently shown parallelism between lysosomal lesions and histopathological and functional alterations in animal kidneys [52, 53]. Thus lysosomal alterations are a significant index, if not a step, in aminoglycoside-induced nephrotoxicity.

NOTE ADDED IN REVISION

While this paper was being reviewed, Hostetler and Hall [54, 55] reported that aminoglycoside antibiotics inhibit *in vitro* the activity of lysosomal phospholipases A and C from the liver and kidney towards the synthetic phospholipid dioleoylphosphatidylcholine.

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