Inhibition of Lysosomal Phospholipases by Aminoglycoside Antibiotics: In Vitro Comparative Studies

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Aminoglycoside antibiotics induce an early and characteristic lysosomal phospholipidosis in cultured fibroblasts and in kidney tubular cells. We have recently demonstrated an inhibition of lysosomal phospholipases A1 and A2 by gentamicin and amikacin in vitro. In vivo, gentamicin decreases the activity of phospholipase A2 (Laurent et al., Biochem. Pharmacol. 31:3861–3870, 1982). In the present study, we examined 14 aminoglycosides for in vitro inhibition of phospholipases.

To mimic the situation prevailing in lysosomes, the enzymatic activities were assayed with phospholipid vesicles (liposomes) with a composition similar to that of lysosomal phospholipids (phosphatidylcholine, sphingomyelin, phosphatidylinositol, cholesterol; 4:4:3:5.5, molar ratio). We measured the hydrolysis of 1-palmitoyl-2-[1-14C]oleoyl phosphatidylcholine contained in the liposomes by a soluble fraction of highly purified lysosomes isolated from rat liver. Similar IC50s (concentrations causing 50% inhibition of enzymatic activity) were observed for dibekacin, gentamicin (with no major difference between C1, C12, or C2), netilmicin, tobramycin, and kanamycin B. Sisomicin was slightly more inhibitory. Kanamycin A, N1-(t-4-amino-2-hydroxy-1-oxobutyl)dibekacin, and amikacin showed increasing IC50s. Streptomycin caused the least inhibition. Octa- and tetramethylkanamycin A are much less inhibitory than the parent drug. These results point to the number, the nature, and the respective positions of the cationic groups as essential determinants in causing inhibition of phospholipid breakdown. The binding of three aminoglycosides (gentamicin, amikacin, streptomycin) to the liposomes at pH 5.4 was also measured by gel permeation and was found to be related to the respective inhibitory potency of each drug. Insofar as lysosomal phospholipidosis is an early sign of intoxication by aminoglycosides, these results may serve as a basis for the development or screening of less toxic compounds in this class of antimicrobial agents.

Aminoglycoside antibiotics are nephrotoxic (2). The earliest and most conspicuous alteration induced by these drugs in kidney ultrastructure is the accumulation of lamellar, osmiophilic material (myeloid bodies) in the lysosomes of proximal tubular cells (22). The characteristic periodic pattern of myeloid bodies, made of concentric layers at a 5-nm distance, suggests that they are made of polar lipids. In cultured fibroblasts exposed to gentamicin (3), as well as in the kidney cortex of treated animals (5, 10, 24), a significant increase in total lipid phosphorus has been observed. The studies with fibroblasts (3) have demonstrated a direct correlation between the accumulation of myeloid bodies in lysosomes and the increase of cell lipid phosphorus. Moreover, each micromole excess of phospholipid was shown to correspond to a volume of 1.8 μl of myeloid bodies, a figure similar to that found for multilamellar liposomes (artificial structures made of concentric layers of phospholipids [36]).

Accumulation of phospholipids in the lysosomes of either fibroblasts or kidney proximal tubular cells has been explained by a decrease in the activities of lysosomal phospholipase A1 and sphingomyelinase after aminoglycoside administration (3, 5, 6, 24, 39). It was also reported that aminoglycosides accumulate in the lysosomes (18, 35, 40). Recently, another group of investigators and ourselves (15, 24) showed independently that lysosomal phospholipases are inhibited in vitro by aminoglycosides. The concentrations needed to produce this inhibition (0 to 500 μg/ml) are in the range of those observed in the lysosomes of fibroblasts (3) or proximal tubular cells (24, 29). This finding has rationalized the concept that aminoglycosides induce a
lysosomal phospholipidosis (3, 20).

In this paper, we compare a large number of aminoglycosides for their ability to inhibit lysosomal phospholipases in vitro. The results of this study may serve as a basis for a more complete understanding of the toxicological behavior of these drugs in vivo.

**MATERIALS AND METHODS**

**Preparation of liposomes.** Liposomes (10 μmol of phospholipid per ml) were prepared in 4 mM sodium acetate buffer (pH 5.4) as described earlier (24), with egg yolk phosphatidylcholine, bovine brain sphingomyelin, wheat germ phosphatidylisinitol, and cholesterol (in a molar ratio of 4:3:5:5.5) and 140 μCi of labeled phosphatidylcholine (1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine) per mmol of phosphatidylcholine. Liposome preparations were stored at 4°C under nitrogen and used within a week.

**Enzyme source.** We used a soluble fraction of liver lysosomes, isolated and ruptured by hypotonic shock and centrifuged as described previously (33, 38). Typical enrichment was 40- to 50-fold, on the basis of the specific activity of β-N-acetylhexosaminidase (assay method in reference 34) and phospholipase A₂ (see below), with a yield of 15 to 20% of the total liver activity. The phospholipid content of this soluble fraction was 0.12 μmol per mg of protein as compared with 0.52 μmol/mg for total lysosomes and 1.6 μmol/mg for the insoluble lysosomal pellet. The latter pellet contained less than 10% of the phospholipase activities present in total lysosomes, as was also found independently by others (23).

**Measurement of labeled phosphatidylcholine breakdown.** Liposomes were mixed with aminoglycosides (sulfate salts) in varying amounts (0 to 65 μg of base per μmol of phospholipid), the final concentration of phospholipid being in all cases ca. 9 μmol/ml. It was checked by paper chromatography that the addition of the aminoglycosides did not change significantly the pH of the mixture (5.4). After 1 h of preincubation at 37°C, the liposome-aminoglycoside mixture (usually 50 μl) was diluted twice with an equal volume of enzyme preparation (final protein concentration, 250 μg/ml). After 30 min of incubation at 37°C, the reaction was stopped by the addition of 2.5 volumes of methanol, and the mixture was separated into two samples which were dried under nitrogen. The substrate and reaction products were separated by thin-layer chromatography, and their radioactivity was measured by scintillation counting, as described earlier (24). Usually the distribution of the radioactivity in the two samples at the corresponding spots did not differ by more than 1% of the total radioactivity, and the data were pooled. In preliminary experiments, the identity of each labeled product was checked in at least two different chromatographic systems (21).

**Aminoglycoside binding to liposomes.** The amount of antibiotic associated with the phospholipid vesicles was estimated by gel permeation, according to a procedure described previously (24). Briefly, aminoglycosides were mixed with liposomes (10 μmol of phospholipids) in a volume of 1 ml in a molar ratio of 1 or 2 mol of drug per 100 mol of phospholipids (see Table 3 for values in micrograms of base). After 1 h at 37°C, the mixture was applied to a column (1.6 by 24 cm) packed with Sepharose 4B and eluted at room temperature with 4 mM acetate buffer, pH 5.4. After a first elution, the free antibiotic retained by the gel was washed out with 50 mM cacodylate-150 mM NaCl (pH 7.4). Aminoglycoside and phospholipid associated with all fractions were measured (see below). The recoveries of antibiotics as compared with the amounts applied to the column were 101 ± 11%; recoveries of phospholipids were 101 ± 4%.

**Analytical assays.** Gentamicin and amikacin were assayed by fluorometry after reaction with fluorescamine as described earlier (24, 42). Streptomycin was assayed by microbiological assay in 0.003 M phosphate buffer-0.15 M NaCl (pH 8.0) by the cylinder plate method with Bacillus subtilis as a test organism (12). It was checked that the liposomes did not interfere with the assay under these conditions, which minimize the liposome-aminoglycoside interactions (24).

Proteins were assayed according to Lowry et al. (27), and total lipid phosphorus was assayed by the Bartlett procedure (4) after complete mineralization of the samples in 60% perchloric acid at 210°C.

**Antibiotics.** Gentamicin, netilmicin, and sisomicin were received from Schering Corp. (Kiel, West Germany, N.J.). Amikacin and tobramycin were a gift from Bristol Benelux (Brussels, Belgium) and E. Lilly Belgum (Brussels, Belgium), respectively. These antibiotics were provided as the sulfate salt preparations under clinical practice. Dibekacin and kanamycin A (both sulfates) were obtained from Continental Pharma, S.A. (Brussels, Belgium). Kanamycin B sulfate and HABA-dibekacin sulfate [N₁-[1,4-amino-2-hydroxy-1-oxobutyl]dibekacin] were obtained from Bristol Laboratories (Syracuse, N.Y.). The components of gentamicin (C₁, C₄a, and C₅) were isolated by column chromatography on silica gel according to Cooper et al. (8). The free bases were converted into the sulfate salts and freeze-dried. The identity and purity were checked by ascending thin-layer chromatography of 10-μg samples on precoated silica gel plates 60 (E. Merck, AG, Darmstadt, Germany), using the lower phase of methanol-chloroform-25% ammonium hydroxide (1:1:1) as a solvent system (45). Only one spot was visible after detection with ninhydrin, at Rₚ-values of 0.37, 0.30, and 0.25 for the C₁, C₄, and C₅ components, respectively. The preparation of tetra-N-methyl- and octa-N-methylkanamycin A was reported by Claes and Vanderhaeghe (7). These derivatives are devoid of antibacterial activity. The free bases were converted to the sulfate salts by titration to pH 5.4 with H₂SO₄.

All experiments were done with the sulfate salt of each antibiotic. To make comparisons between antibiotics easier, all concentrations are, however, expressed in free-base equivalents. The percentage of antibiotic in each preparation was that communicated by the supplier for gentamicin, amikacin, tobramycin, dibekacin, netilmicin, sisomicin, kanamycins A and B, and HABA-dibekacin, or it was that determined by titration for tetra- and octamethylkanamycin A, or it was measured by independent microassay of sulfate and water content for the components of gentamicin (assay performed by Analytische Laboratorium, H. Malisse und G. Reuter, Engelskirchen, West Germany).

**Other reagents and chemicals.** d-Glucosamine (hy-
dichloride salt) was purchased from E. Merck AG. Natural glycerophospholipids (grade I) were obtained from Lipid Products (Nr. Redhill, England). Bovine sphingomyelin and cholesterol were purchased from Sigma Chemical Co. (St. Louis, Mo.). Labeled phosphatidylcholine (1-palmitoyl-2-[1-14C]oleoyl-sn-glycero-3-phosphocholine) was obtained from the Radiochemical Centre (Amersham, Bucks, England). The purity of labeled and nonlabeled lipids was checked by thin-layer chromatography and found to be higher than 98 to 99%, in accordance with the supplier's specifications. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Inc., (Uppsala, Sweden).

RESULTS

Figure 1 shows the catabolic pathways of phosphatidylcholine by lysosomal enzymes. Studies with liver lysosomes and kidney homogenates have shown that the initial degradation is predominantly carried out by phospholipase A1 (EC 3.1.1.32). Phospholipases A2 and C are of lesser quantitative importance (23, 24).

In this study, we have used a substrate labeled at the fatty acid in position 2 (unsaturated). The release of labeled lysophosphatidylcholine is therefore a direct measure of phospholipase A1. Figure 2c shows that this activity is optimal at pH 5.4 and that the rate of release of the product is almost constant up to 40 min of incubation (ca. 20% hydrolysis of the substrate). The specific activity was typically ca. 40 nmol of 2-[1-14C]oleoyl lysophosphatidylcholine released per min and per mg of protein. Release of labeled fatty acid was also detected, with an optimum at pH 5.4 (Fig. 2a). Specific activity at this pH was typically 13 nmol of [1-14C]oleic acid released per min and per mg of protein. Release of labeled oleic acid may result from the action of phospholipase A2 or β-lysophospholipase or both. Under conditions similar to those used here, we showed previously that two-thirds of this release is due to phospholipase A2 (24). Activity of phospholipase C (release of labeled diacyl- or monoacylglycerol [13]) was not detected under our conditions.

Both the release of β-lysophosphatidylcholine and that of fatty acid are inhibited by aminoglycosides. This inhibition was investigated in 30-min incubation experiments (after a 60-min preincubation of liposomes and drug). In preliminary experiments, we found that the release of fatty acid, which is not linear for more than 20 min, is inhibited to the same extent after 20 or 30 min of incubation. Dose-response curves of this inhibition on the release of lysophosphatidylcholine and of fatty acid are shown in Fig. 3 for gentamicin, amikacin, and strepto-

FIG. 1. Degradation pathways of phosphoglycerides in lysosomes, based on studies with phosphatidylcholine (11, 13, 23, 24). The molecule represented schematically on top is phosphatidylcholine. The asterisk shows the position of the radioactive label in the substrate used in the present study (1-palmitoyl-2-[1-14C]oleoyl phosphatidylcholine). The thickness of the arrows indicates the relative contributions of each pathway under our experimental conditions, as determined in a previous study (24). The broken line (phospholipase C) indicates no activity detected. The position of the label does not allow the study of the degradation of α-lysophosphatidylcholine (dotted line). Abbreviations: Ch, choline; FA, saturated fatty acid (palmitate); uns. FA, unsaturated fatty acid (oleate); A1, phospholipase A1; A2, phospholipase A2; C, phospholipase C.
Gentamicin. Above a concentration of ca. 75 μg/ml for gentamicin and 150 μg/ml for amikacin, the release of lysophosphatidylcholine (phospholipase A₁ activity) is almost completely abolished. The release of fatty acid is also strongly decreased, which indicates an inhibition of phospholipase A₂. Streptomycin is a much less potent inhibitor, and about 200 μg/ml was required to inhibit the release of β-lysophosphatidylcholine and of oleic acid to 50%. We checked that the SO₄²⁻ added with the aminoglycosides had no inhibitory effect by running experiments with the corresponding amounts of Na₂SO₄. We also checked that an aminated monosaccharide such as D-2-deoxy-2-aminoalglucose had no effect up to 800 μg/ml (ca. 4 mM as compared with 90% inhibitory concentration of ca. 0.16 mM for gentamicin) when used as the neutralized salt form. In other experiments with crude preparations of kidney lysosomes (44), we found qualitatively similar results for gentamicin and amikacin. Lower specific activities and contamination by endogenous phospholipids made, however, the assays more difficult and less reproducible. We did not attempt to use delipidated enzyme preparations, since this treatment causes a striking decrease in phospholipase A₁ activity (23).

Complete dose-response curves were established for all other aminoglycosides. Their shape was similar to those of gentamicin, and the drug concentrations causing 50% (IC₅₀) and 90% (IC₉₀) inhibitions of the release of labeled lysophosphatidylcholine and of fatty acid are shown in Table 1. For those compounds tested in three or more independent experiments, only HABA-dibekacin, kanamycin A, amikacin, and streptomycin show consistently higher IC₅₀ and IC₉₀ than gentamicin, with streptomycin being the least inhibitory of all drugs tested. Gentamicin C₁₈ shows a significant difference only for the release of fatty acid. Only sisomicin is significantly more inhibitory than gentamicin for three out of four values. The IC₅₀ and IC₉₀ of structurally related antibiotics were systematically compared, and we found the following significant differences. (i) According to the number of amino groups, kanamycin B < kanamycin A (P < 0.05 for all values). (ii) After the displacement of the N₁ amino group away from the ring, dibekacin < HABA-dibekacin (P <
0.05 for IC<sub>90</sub>s and P < 0.005 for IC<sub>50</sub>s) and kanamycin A < amikacin (P < 0.05 for IC<sub>50</sub> of phospholipase A<sub>1</sub> and P < 0.005 for all other values). (iii) After the conversion of the N-1 primary amine into a secondary amine, sisomicin < netilmicin (P < 0.05 for all values except for the IC<sub>50</sub>s of the release of fatty acid, the difference of which is not significant). Methylation of the four amines of kanamycin A decreases very strongly the inhibitory potency, and no IC<sub>50</sub>s could be determined for those derivatives. Deoxygenation of the 2',6'-diaminoglucose moiety induces only moderate change (kanamycin B versus dibekacin, P > 0.1 for all values except for the IC<sub>90</sub>s of phospholipase A<sub>1</sub>).

In a previous publication (24) we showed that aminoglycosides bind to phospholipid bilayers which contain phosphatidylinositol, and we suggested that this binding is responsible for the inhibition of the phosphatidylcholine breakdown present in the bilayer. To check on this we prepared liposomes without phosphatidylinositol, and we looked at the inhibitory effect of gentamicin on phosphatidylcholine breakdown under these conditions. Sphingomyelin was also omitted after we found liposomes made of phosphatidylcholine, sphingomyelin, and cholesterol (4:7:5.5) to be unstable in our conditions. Table 2 shows that the omission of phosphatidylinositol from the liposomes reduces the activity of phospholipase A<sub>1</sub> and still more the activity of phospholipase A<sub>2</sub> or β-lysophospholipase or both. Yet no inhibitory action of gentamicin is seen at a concentration equal to the IC<sub>50</sub> measured with phosphatidylinositol-containing liposomes. Table 2 also shows that liposomes without phosphatidylinositol do not appreciably bind gentamicin.

The influence of the binding of aminoglycosides to liposomes on the inhibition of phospholipase activity was further explored with gentamicin, amikacin, and streptomycin at drug concentrations spanning the range at which partial inhibition of phospholipid degradation is evidenced. Table 3 shows that streptomycin...
TABLE 1. Effect of various aminoglycosides on phosphatidylcholine breakdown

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>Concentration (µg/ml)</th>
<th>β-Lysophosphatidylcholine</th>
<th>Fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Dibekacin</td>
<td>44 ± 10</td>
<td>67 ± 7</td>
<td>50 ± 8</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>48 ± 2</td>
<td>65 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>52 ± 9</td>
<td>73 ± 6</td>
<td>54 ± 6</td>
</tr>
<tr>
<td>Gentamicin component C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>42 ± 2</td>
<td>67 ± 1</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>Gentamicin component C&lt;sub&gt;1a&lt;/sub&gt;</td>
<td>46 ± 4</td>
<td>71 ± 1</td>
<td>66 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gentamicin component C&lt;sub&gt;2&lt;/sub&gt;</td>
<td>45 ± 4</td>
<td>64 ± 6</td>
<td>68.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>53 ± 3</td>
<td>77 ± 8</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>54 ± 4</td>
<td>77 ± 5</td>
<td>56 ± 7</td>
</tr>
<tr>
<td>Netilmicin</td>
<td>57 ± 3</td>
<td>78 ± 6</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>69 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>105 ± 14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>69 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HABA-dibekacin</td>
<td>78.5 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>79 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amikacin</td>
<td>85 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>143 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>96 ± 4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Octamethylkanamycin A</td>
<td>92</td>
<td>&gt;200</td>
<td>104</td>
</tr>
<tr>
<td>Tetramethylkanamycin A</td>
<td>103</td>
<td>&gt;200</td>
<td>167</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>238 ± 30°</td>
<td>&gt;333&lt;sup&gt;c&lt;/sup&gt;</td>
<td>195 ± 38°</td>
</tr>
</tbody>
</table>

* a The concentrations causing 50% (IC<sub>50</sub>) and 90% (IC<sub>90</sub>) inhibitions of the release of labeled β-lysophosphatidylcholine and oleic acid were measured in dose-response experiments similar to those shown in Fig. 3. Each figure is the mean of the IC<sub>50</sub> and IC<sub>90</sub> determined from independent curves (± standard deviation). For the IC<sub>90</sub> a and IC<sub>90</sub>b higher than those of gentamicin, the statistical significance of the difference was analyzed by the Student’s t test.

b <i>P</i> < 0.05.

c <i>P</i> < 0.001.

and, to a lesser extent, amikacin bind consistently less than gentamicin. Figure 4 shows the relation between the amount of drug bound to liposomes and the inhibition of phospholipase A<sub>1</sub> with streptomycin, amikacin, and gentamicin.

**DISCUSSION**

The relevance of the present report to the toxicity of aminoglycosides in vivo (5, 9, 17, 22, 25) or in cultured cells (3, 41) stems from the fact that these polycationic drugs concentrate in lysosomes (18, 19, 29, 35, 40). Lysosomes are rich in negatively charged phospholipids (28, 37) and display an acidic pH (30, 32). Electrostatic complexes between aminoglycosides and phospholipids are therefore likely to spontaneously form in lysosomes (1, 26, 43). This binding could decrease the activity of lysosomal phospholipases, which would explain the accumulation of myeloid bodies in lysosomes (3, 5, 9, 17, 22, 25) and the concomitant increase of lipid phosphorus observed after aminoglycoside treatment (3, 5, 10, 24, 41). Inhibition of lysosomal phospholipase A<sub>1</sub> has been demonstrated in genticmicin-treated animals (5, 24).

TABLE 2. Activities of phospholipase A<sub>1</sub> and phospholipase A<sub>2</sub> and β-lysophospholipase towards phosphatidylinositol-containing and phosphatidylinositol-free liposomes: binding of gentamicin and the effect on phospholipid hydrolysis

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Phospholipase A&lt;sub&gt;1&lt;/sub&gt;</th>
<th>Phospholipase A&lt;sub&gt;2&lt;/sub&gt; plus β-lysophospholipase</th>
<th>Aminoglycoside bound to liposome (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With gentamicin (49 µg/ml)</td>
<td>Control</td>
</tr>
<tr>
<td>With phosphatidylinositol&lt;sup&gt;4&lt;/sup&gt;</td>
<td>48.7 ± 1.4</td>
<td>29.2 ± 1.7</td>
<td>14.9 ± 1.5</td>
</tr>
<tr>
<td>Without phosphatidylinositol&lt;sup&gt;4&lt;/sup&gt;</td>
<td>35.6 ± 1.7</td>
<td>34.0 ± 5.4</td>
<td>1.6 ± 0.5</td>
</tr>
</tbody>
</table>

* a Given as the nanomoles of β-lysophosphatidylcholine released per minute per milligram of protein ± the standard deviation (n = 3).

b Given as the nanomoles of oleic acid released per minute per milligram of protein ± the standard deviation (n = 3).

c Percentage of added aminoglycoside (see Table 3).

d Molar ratio of phosphatidylcholine/sphingomyelin/phosphatidylinositol/cholesterol was 4:4:3:5.5.

e Molar ratio of phosphatidylcholine/cholesterol was 11:5.5.
TABLE 3. Binding of aminoglycosides to liposomes

<table>
<thead>
<tr>
<th>Aminoglycoside</th>
<th>Molar ratio of aminoglycoside/phospholipid in preincubation*</th>
<th>Aminoglycoside conc in preincubation (µg/ml)</th>
<th>Molar ratio of aminoglycoside/phospholipid in liposome peak*</th>
<th>Aminoglycoside bound to liposomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>1:100</td>
<td>49</td>
<td>1.134:100</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>2:100</td>
<td>49</td>
<td>1.242:100</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>49</td>
<td>1.134:100</td>
<td>90</td>
</tr>
<tr>
<td>Amikacin</td>
<td>1:100</td>
<td>62</td>
<td>0.876:100</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>2:100</td>
<td>124</td>
<td>1.570:100</td>
<td>69</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1:100</td>
<td>62</td>
<td>0.135:100</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>2:100</td>
<td>124</td>
<td>0.607:100</td>
<td>26</td>
</tr>
</tbody>
</table>

* The amount of antibiotic associated to the phospholipid bilayer was estimated by gel permeation of aminoglycoside-liposome mixtures on Sepharose 4B (see the text).

FIG. 4. Relationship between binding and inhibition of phospholipase A<sub>2</sub> activity for gentamicin (●, ○), amikacin (■), or streptomycin (▲). This graph is based on binding data from Tables 2 and 3 and from inhibition experiments similar to those in Fig. 3. The closed symbols refer to experiments done with phosphatidylinositol-containing liposomes. The open circle refers to the use of liposomes without phosphatidylinositol (see Table 2). Bars refer to the standard deviations (three or more experiments) or ranges of values (two experiments).
This study extends to a larger number of aminoglycosides our observation (24) that gentamicin inhibits lysosomal phospholipases in vitro. Moreover, we demonstrate a relationship between the potency of three aminoglycosides to inhibit these enzymes and their relative binding to liposomes rich in phosphatidylinositol. The interesting feature demonstrated by the present data is that the catabolism of a nonacidic phospholipid present in the bilayer, namely, phosphatidylcholine, is impaired, although phosphatidylcholine alone is unable to bind much aminoglycoside. Phosphatidylcholine is a major phospholipid, and inhibition of its breakdown is expected to give rise quickly to lysosomal overproduction (3, 24). We are currently investigating the influence of aminoglycosides on the breakdown of the other major phosphoglycerides, such as phosphatidylethanolamine, which are most likely degraded by the same enzymes as those shown in Fig. 1 (11, 23).

The number, the nature, and the respective position of the amino groups in the molecule seem to play a major role in the inhibitory activity of aminoglycosides towards phospholipases. Thus, gentamicin, tobramycin, dibekacin, and kanamycin B, which all have five amino groups similarly displayed on the molecule, have a common behavior, despite other significant structural differences among them. Our results show that methylation of a single primary amine has no or little influence (3′ amino group: tobramycin, kanamycin B, and dibekacin versus the gentamicins C or sisomicin; 6′ amino group: gentamicins C₁ and C₂ versus gentamicin C₁₄). Ethylation of the 1 amino group in deoxystreptamine is however effective (sisomicin versus netilmicin). On the other hand, methylation of all amino groups drastically decreases the inhibitory potency (kanamycin A versus tetra- or octa-N-methylkanamycin A). The removal of an amino group (kanamycin A versus kanamycin B) or its displacement away from the ring structure (amikacin versus kanamycin A; HABA-dibekacin versus dibekacin) also results in a significant decrease of inhibition. Streptomycin is also polycationic, but is not very inhibitory. The nature of the cationic groups and the structure of the molecule are quite different from those of the other drugs considered here. Finally, the respective behaviors of streptomycin and of the methyalted derivatives of kanamycin A show that the inhibitory potency of aminoglycosides towards phospholipases is not simply paralleled by their antibacterial activity.

Hostetler and Hall (14, 15) have observed a similar ranking for the inhibitory potencies of dibekacin, gentamicin, and amikacin on the hydrolysis of dioleoylphosphatidylcholine by liver or kidney lysosomal extracts. With this substrate, and perhaps too because of the use of delipidated enzyme preparations, the activity of the phospholipases A is much lower, and the reported IC₅₀s were considerably higher than those presented here (e.g., for gentamicin, 4- and 40-fold for liver and kidney enzyme, respectively). The IC₅₀s could not be determined. Tobramycin was also reported to be significantly more inhibitory than dibekacin. The published figures of antibacterial potency of either drug suggest, however, that tobramycin base was compared to dibekacin sulfate. Part of the difference may thus be due to a rise in pH in the incubation mixture, especially with kidney enzymes which show very steep pH dependence curves (15).

The results of our in vitro comparative studies should not be extended to the in vivo situation without caution. As pointed out repeatedly (15, 20, 29, 41), the severity of the lysosomal alterations induced in the kidney by each aminoglycoside is due to the combination of (i) the ability of the drug to be taken up by the proximal tubular cells and to accumulate in lysosomes and (ii) its intrinsic potency to inhibit phospholipid breakdown. Thus, tobramycin and gentamicin could not be distinguished for toxicity in cultured fibroblasts where their accumulation level is similar (41). On the other hand, tobramycin is less accumulated by the kidney cortex of rats than gentamicin (31) and induces less lysosomal alteration. Histopathological studies, examining necroses and regeneration of tubular cells, have demonstrated a relative nephrotoxicity score of gentamicin > tobramycin > amikacin at low doses (16). This pattern is in accordance with our data (Table 1), taking into account the lesser uptake of tobramycin by the rat kidney cortex (31, 39). Similarly, streptomycin is almost not nephrotoxic. A more systematic survey is, however, necessary to unambiguously establish a relationship between the inhibition of phospholipid breakdown and the development of histopathological and functional alterations typical of aminoglycoside toxicity (9, 16, 17).

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LITERATURE CITED

1. Alexander, A. M., I. Gonda, E. S. Harpur, and J. B. Kayes. 1979. Interaction of aminoglycoside antibiotics with phospholipid liposomes studied by microelectropho-
The purpurosamines, a new class of naturally occurring 2,6-diaminomonosaccharides, p. 481-491.


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