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EFFECT OF ACIDIC PHOSPHOLIPIDS ON THE ACTIVITY OF LYSOSOMAL PHOSPHOLIPASES AND ON THEIR INHIBITION BY AMINOGLYCOSIDE ANTIBIOTICS—I

BIOCHEMICAL ANALYSIS

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Abstract—Aminoglycoside antibiotics accumulate in lysosomes of kidney and cultured cells and cause an impairment of phospholipid catabolism which is considered to be an early and significant step in the development of their toxicity. Using liposomes, we previously demonstrated that the activity of lysosomal phospholipases A_1 and A_2 towards phosphatidylcholine was markedly enhanced by the inclusion of phosphatidylinositol in the bilayer, and that gentamicin impaired this activity by binding to phosphatidylinositol. Since gentamicin-induced inhibition was inversely related to the amount of phosphatidylinositol included in the liposomes, we proposed that gentamicin impairs activity of phospholipases by decreasing the quantity of available negative charges carried by the bilayer surface (Mingeot-Leclercq *et al.*, *Biochem Pharmacol* **37**: 591–599, 1988). We now extend these observations to phosphatidylserine and phosphatidic acid, and compare the inhibition caused by gentamicin, amikacin and streptomycin towards lysosomal phospholipases on the hydrolysis of phosphatidylcholine in the presence of each of these acidic phospholipids. Inclusion of phosphatidic acid in liposomes, and, to a lesser extent, phosphatidylserine, caused a larger increase in phospholipases activity than phosphatidylinositol. In parallel, the three aminoglycosides tested were found less inhibitory towards phospholipases activity measured on phosphatidic acid- or phosphatidylserine-containing liposomes than was previously observed with phosphatidylinositol, even though equilibrium dialysis experiments failed to demonstrate significant difference in binding parameters of the drug towards each of these liposomes populations. Yet, as for phosphatidylinositol-containing liposomes, the inhibition was inversely related to the amount of phosphatidic acid or phosphatidylserine included in the bilayer and the inhibitory potency of the three drugs was consistently gentamicin > amikacin > streptomycin with the three types of negatively-charged liposomes used. We conclude that impairment of lysosomal phospholipases activity towards phosphatidylcholine included in negatively-charged membranes by aminoglycoside antibiotics is dependent upon drug binding to the bilayer, but that it is modulated by the nature of the acidic phospholipid that binds the drug as well as by that of the drug itself. A companion paper (Mingeot-Leclercq *et al.*, *Biochem Pharmacol* **40**: 499–506, 1990) will examine by computer-aided conformational analysis the parameters (drug–phospholipid energy of interaction, position of the drug in a monolayer and its accessibility to the aqueous phase) which may be important for these effects.

Aminoglycoside antibiotics are useful in the treatment of infections caused by Gram(−) bacteria, but show a narrow therapeutic index because of oto- and nephrotoxic reactions [1–3]. These drugs are taken up by the proximal tubular cells of renal cortex and sequestered in lysosomes [4, 5], which display early alterations consisting in the inhibition of the activities of phospholipases A (measured towards phosphatidylcholine), sphingomyelinase, and phosphatidylinositol phospholipase C ([6]; P. Lambricht and G. Laurent, personal communication). A concomitant accumulation of myelin-like figures in lysosomes, associated to an enlargement of their size and a decrease of their buoyant density, has also been observed [5, 7–9]. These alterations are followed by the development of tubular necrosis and nephrotoxicity, but the underlying mechanism(s) relating these phenomenons remain still uncovered (see discussion in Refs 10 and 11). Recently, it was shown that polyaspartic acid, which completely protects against aminoglycoside-induced nephrotoxicity [12, 13], also protects against the development of the early lysosomal alterations described above [14, 15], suggesting that the inhibition of lysosomal phospholipase activities by aminoglycosides is indeed a key factor in the cascade of events leading to nephrotoxicity.

Previous studies have shown that aminoglycoside antibiotics inhibit the activities of lysosomal phospholipases A and C *in vitro* when the substrate is organized as negatively-charged bilayers or micelles [6, 16, 17]. Binding of the drug to the phospholipid bilayer is critical in the inhibition of phospholipases A [18, 19], and we have recently proposed that aminoglycosides actually decrease the available

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negative charge required in the bilayer for optimal activity [20]. Indeed, the activity of lysosomal phospholipases A_1 and A_2 towards phosphatidylcholine included in liposomes markedly increases when the phosphatidylinositol content of the vesicles is raised from 10 to 30% of the total phospholipid content [20], i.e. in a range found in most natural membranes for total negatively-charged phospholipids [21, 22]. Addition of gentamicin, taken as a typical aminoglycoside, causes a concentration-dependent decrease in these activities, but this effect is inversely proportional to the amount of phosphatidylinositol present in the bilayer [20]. In the present paper, we further characterize the influence exerted by negative charges carried by the phospholipid bilayer on the activity of lysosomal phospholipases towards phosphatidylcholine and on their inhibition by aminoglycosides. Thus, we examine the influence of the nature of the acidic phospholipid included in liposomes (phosphatidylinositol vs phosphatidylserine vs phosphatidic acid) on the activity of these enzymes, and measure the inhibitory potency of gentamicin in correlation with its binding towards the three types of liposomes, as measured by equilibrium dialysis. We also compare gentamicin with two other aminoglycosides, namely amikacin and streptomycin which are less nephrotoxic [1, 3, 8] and which, in previous studies, were found less inhibitory towards phospholipases measured with phosphatidylinositol-containing liposomes [6, 19]. This approach, combined with computer-aided conformational studies reported in a companion paper [23], may enhance our molecular understanding of the parameters which govern the activity of lysosomal phospholipases and influence the inhibitory potency of aminoglycoside antibiotics towards lysosomal phospholipases.

MATERIALS AND METHODS

Preparation of liposomes. Unless stated otherwise, sonicated liposomes were prepared from cholesterol, egg yolk phosphatidylcholine, bovine brain sphingomyelin and one of the following acidic phospholipids, namely wheat germ phosphatidylinositol, bovine spinal cord phosphatidylserine, or phosphatidic acid (made from egg lecithin by the action of phospholipase D). The phospholipid : cholesterol molar ratio was set at a value of 2 : 1, and the phosphatidylcholine content was kept constant at 4 moles per 11 moles of phospholipids throughout. The negative charge of the liposomes was varied by adding increasing amounts of one acidic phospholipid, from 0 up to 6 moles, whereas the sphingomyelin content was correspondingly decreased from 7 (for neutral liposomes) to 1 mole per 11 moles of phospholipids. Labelled phosphatidylcholine (1-palmitoyl, 2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) was added for enzymatic and partition studies (140 and 10 mCi/mol of phosphatidylcholine, respectively). Liposomes were routinely prepared in acetate buffer at pH 5.4 as described earlier [6] except that the concentration of the buffer was 40 mM instead of 4 mM in order to keep the pH constant in spite of the wide variations in acidic phospholipid content. The final lipid concentration was 10 g/L. Liposomes were stored under nitrogen and used within a week.

Determination of size of liposomes. The apparent average diameter of the liposomes was evaluated by quasielastic light scattering spectroscopy [24] using a Coulter® Nano Sizer™ (Coulter Electronics Ltd, Luton, U.K.). Fluctuation of light scattering was measured at an angle of 90°, and monodisperse latex particles of 100 and 800 nm diameter (Dow Chemical Co., Midland, MI) were used as control.

Enzymatic studies. The activity of lysosomal phospholipase A_1 (phosphatidate 1-acylhydrolase, EC 3.1.1.32) and the combined activities of phospholipase A_2 (phosphatidate 2-acylhydrolase, EC 3.1.1.4) and of betalysophospholipase (lysolecithin 2-acylhydrolase, EC 3.1.1.-) towards 1-palmitoyl, 2-[1-^{14}C]oleoyl phosphatidylcholine included in the liposomes were determined by measuring the release of [^{14}C]lysophosphatidylcholine and of [1-^{14}C]fatty acid, respectively, upon exposure of the liposomes to a soluble fraction of purified rat liver lysosomes, as described previously [6, 18, 20].

Determination of liposomes surface charge. The variation of surface charge of liposomes was estimated by determining their partition in an aqueous two-phases polymer system [25]. The system used here was obtained by mixing 1.4 g of Dextran T500 20% (w/w) to 0.7 g of polyethyleneglycol 4000 40% (w/w), and then adding 1.9 g of 0.15 M NaCl and citrate (4.5 mM) phosphate (11 mM) buffer, pH 5.4, 50 : 50 (v/v). The final system consisted therefore of 7% (w/w) Dextran T500, 7% (w/w) polyethyleneglycol 4000, NaCl (35.6 mmol/kg) and phosphate (2.64 mmol/kg), buffered at pH 5.4. Phosphate ions created a 2 mV potential difference (measured with a voltmeter of high sensitivity) between the two phases due to their preferential distribution in the dextran-rich (lower) phase. The partition behaviour of negatively-charged liposomes was determined by using preparations containing trace amounts of ^{14}C-labelled phosphatidylcholine, as described by Sharpe [26]. Briefly, 1 mL of liposomes suspension was added to the 4 g of the two-phases system, the phases were mixed at room temperature by 30 inversions in 5-mL graduated syringes, separated by spinning the samples at 34 *g* for 5 min, and 200 μL from the centre of each phase were collected for radioactivity measurement.

Binding studies. The binding of gentamicin to liposomes containing an increasing proportion of one acidic phospholipid (phosphatidylinositol, phosphatidylserine or phosphatidic acid) was studied by equilibrium dialysis using a Dianorm® apparatus (Dianorm Geräte, München, F.R.G.), consisting of sets of 0.2-mL twin cells made of Teflon® and separated by a Diachema® flat dialysis membrane (M_r cut off 5000). Liposomes were dispersed in appropriate volumes of acetate buffer 40 mM pH 5.4 in order to obtain liposomes preparations with approx. 250 μmol of acidic phospholipid per mL over the whole range of acidic phospholipid/total lipid ratios investigated (0.061–0.364). Two hundred microlitres of these liposomes preparations were then dialysed against gentamicin (4–40 nmol/200 μL in the same buffer) for 5 hr at 4° under constant rotation of the cell carrier unit (12 rpm). Samples collected from the

cells without liposomes were assayed before and after dialysis by reaction with fluorescamine [27], and the amount of bound gentamicin calculated from the difference. Determination of the binding parameters was made by direct analysis of the Bound vs Free gentamicin concentrations plots, and fitting the experimental points to a hyperbolic equation using the damping Gauss–Newton procedure [28]. The program used is derived from published literature [29] and further information can be obtained from its author (J.P.).

Materials. Gentamicin (sulphate salt) was supplied by Schering-Plough Corporation (Kenilworth, NJ) as "Gentamicin reagent solution" for *in vitro* and investigational use. Like other commercial preparations, the gentamicin samples consisted of a mixture of three main components, C_1, C_{1a} and C_2 (differing by the degree of methylation of the C'6 and N'6 atoms), in a molar ratio of approx. 30:30:40. The inhibitory potency of each of these components towards lysosomal phospholipases *in vitro* does not, however, significantly differ from that of the commercial mixture [18]. Amikacin was the gift from Bristol Belgium (Brussels, Belgium) and was supplied as Amukin® (sulphate salt solution) for clinical use. Streptomycin sulphate was purchased from Laboratorium Wolfs (Antwerp, Belgium). The structural formulae of gentamicin, amikacin and streptomycin have been given in our previous publications [6, 30]. Glycerophospholipids (egg yolk phosphatidylcholine, wheat germ phosphatidylinositol, bovine spinal cord phosphatidylserine and phosphatidic acid made from egg lecithin by the action of phospholipase D) were purchased from Lipid Products (Nr Redhill, U.K.) as grade 1 products, and bovine brain sphingomyelin and cholesterol from the Sigma Chemical Co. (St Louis, MO). Radiolabelled phosphatidylcholine (1-palmitoyl-2[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine; 52 mCi/mmol) was obtained from Amersham International plc (Amersham, U.K.). Dextran T500 was supplied by Pharmacia Fine Chemicals AB (Uppsala, Sweden). Other reagents were obtained from E. Merck AG (Darmstadt, F.R.G.) and were of analytical grade.

RESULTS

In a previous report [20], we showed that the activity of lysosomal phospholipases towards phosphatidylcholine included in liposomes was dependent on the phosphatidylinositol content of the bilayer. In a first step, we have examined whether this observation could be extended to other acidic phospholipids also naturally-occurring in subcellular membranes such as phosphatidylserine or phosphaticid acid [21, 22]. As shown in Fig. 1, the release of labelled lysophosphatidylcholine and of labelled oleic acid (activity of lysosomal phospholipase A_1 and of the sum of phospholipase A_2 and betalysophospholipase, respectively) increased upon addition of each of the three acidic phospholipids in the liposomes roughly over a similar range (10 to about 30% of total phospholipids). The magnitude of this effect, however, varied according to the nature of the acidic phospholipid. Thus, the release of labelled lysophosphatidylcholine was increased up to seven-fold over the value observed for neutral liposomes when phosphatidic acid was included in the bilayer, whereas a maximal stimulation of three–four-fold only was seen with phosphatidylserine or phosphatidylinositol. For the release of labelled fatty acid, addition of phosphatidic acid or phosphatidylserine resulted in an approx. six–seven-fold increase whereas phosphatidylinositol caused only a modest change compared to neutral liposomes. Interestingly enough, activities observed with 9% of phosphatidylinositol, were significantly lower than those recorded with neutral liposomes. Phosphatidylserine and phosphatidic acid did not cause a similar effect. To check that the low activities found with neutral liposomes were not due to the presence of large amounts of sphingomyelin (the concentration of which was decreased when that of acidic phospholipid was increased in order to maintain constant the phospholipid:cholesterol and phosphatidylcholine:total phospholipids ratios), neutral liposomes were prepared with phosphatidylcholine and cholesterol only (4:5.5 mol:mol) and exposed to lysosomal extracts. No significant difference in the activities studied was seen compared to neutral liposomes prepared with sphingomyelin (phosphatidylcholine : cholesterol : sphingomyelin 4 : 5.5 : 7 mol:mol).

In a next step, we compared the inhibitory potency of gentamicin on the activities of lysosomal phospholipases measured towards phosphatidylcholine in liposomes containing a fixed proportion (18%) of each of the three acidic phospholipids studied. The results obtained are shown in Fig. 2. The inhibition of phospholipase A_1 activity was markedly influenced by the nature of the acidic phospholipid, with the strongest inhibition observed with phosphatidylinositol and the lowest with phosphatidic acid. Although less striking, similar differences were also observed for the release of fatty acid. In both cases, the inhibition achieved by gentamicin on the activities measured with phosphatidic acid-containing liposomes was largely uncomplete at the maximal drug concentration investigated.

We reported earlier [20] that the gentamicin-induced inhibition of lysosomal phospholipases activity towards phosphatidylcholine was inversely proportional to the content of the bilayer in phosphatidylinositol. Thus, dose–response curves of the inhibition of lysosomal phospholipases by gentamicin were established for liposomes containing increasing amounts of each of the three acidic phospholipids studied here. Table 1 shows the drug concentration needed to achieve 25% reduction of enzymatic activity (IC_{25}) (this value is shown rather than that causing 50% of activity reduction (IC_{50}) used earlier [18, 19] since inhibition of activity in liposomes containing 18% of phosphatidic acid did not reach 50% at the maximal gentamicin concentration investigated). As for phosphatidylinositol, the inhibitory potency of gentamicin on the activity of lysosomal phospholipases towards phosphatidylcholine was inversely related to the bilayer content in phosphatidylserine or phosphatidic acid. The nature of the acidic phospholipid was, however, also critical over the whole range of contents investigated. IC_{25}

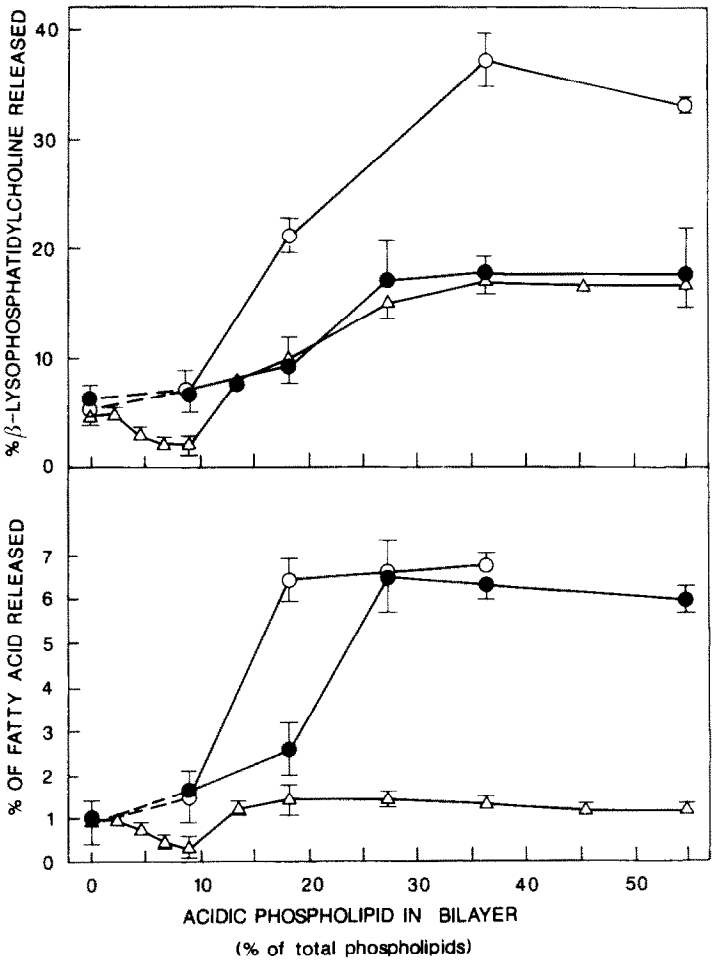

Fig. 1. Degradation of 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine included in liposomes as a function of their content in acidic phospholipid (△, phosphatidylinositol; ●, phosphatidylserine; ○, phosphatidic acid) in the presence of lysosomal extracts at pH 5.4. The amounts of labelled compound released are shown on the ordinates and expressed as the percentage of the total radioactivity present in the assay mixture. The lower part of the diagram shows the release of labelled oleic acid (combined activities of phospholipase A_2 and betalysophospholipase); the upper part shows the release of labelled lysophosphatidylcholine (2-[1^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) (activity of phospholipase A_1). Each symbol with a vertical bar (±SD) refers to the mean of at least three separate experiments.

values systematically increased from phosphatidylinositol- to phosphatidylserine- and to phosphatidic acid-containing liposomes. For the latter, inhibition was very weak and no IC_{25} could be determined in liposomes containing more than 27 and 36% of this acidic phospholipid for the release of lysophosphatidylcholine and of fatty acid, respectively.

We also reported that amikacin and streptomycin are less inhibitory than gentamicin towards lysosomal phospholipases activity measured with phosphatidylinositol-containing liposomes [6, 18]. As for gentamicin, the inhibitory potencies of amikacin and of streptomycin were found inversely related to the content of the bilayer in phosphatidylinositol (data not shown). Table 2 shows that the ranking of inhibitory potencies of the three aminoglycosides was the same for phosphatidylserine- and phosphatidic acid-containing liposomes as reported earlier for phosphatidylinositol-containing liposomes, i.e. gentamicin > amikacin > streptomycin [18]. Moreover, the ranking of the IC_{25} values found for amikacin or streptomycin in function of the nature of the acidic phospholipid included in the bilayer was the same as for gentamicin, i.e. phosphatidylinositol < phosphatidylserine < phosphatidic acid.

Carlier *et al.* [18] observed a direct correlation between the amount of gentamicin, amikacin and streptomycin bound to phosphatidylinositol-containing liposomes and the inhibition of the activity of lysosomal phospholipases towards phosphatidylcholine, as measured in the present study. We therefore measured the binding parameters of gentamicin to liposomes containing increasing amounts of phosphatidylinositol, phosphatidylserine or phosphatidic acid, using the equilibrium dialysis technique. The calculated K_d and B_{max} values are shown in Table 3. The maximal binding capacity of liposomes per

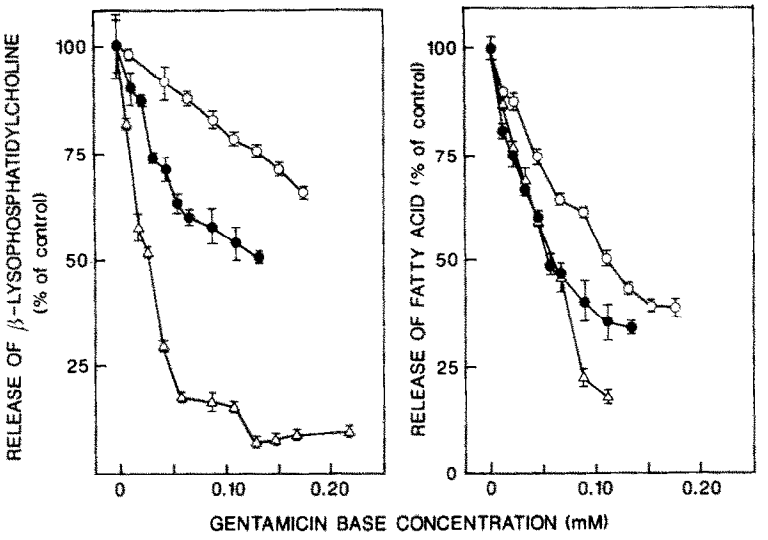

Fig. 2. Effect of gentamicin on the degradation of labelled phosphatidylcholine included in liposomes containing 18% of phosphatidylinositol (△), phosphatidylserine (●) or phosphatidic acid (○) (percentage of total phospholipids) in the presence of lysosomal extracts at pH 5.4. The abscissa shows the actual concentrations of antibiotic in the final assay mixture. The ordinate gives the amount of reaction product as the percentage of that measured in parallel experiments without antibiotic. Part A of the diagram shows the activity of phospholipase A_1, and part B, the combined activities of phospholipase A_2 and β-lysophospholipase, as explained in the legend of Fig. 1.

Table 1. Effect of gentamicin on the degradation of labelled phosphatidylcholine (1-palmitoyl, 2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) included in liposomes containing increasing amounts of one acidic phospholipid (PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid)

Acidic phospholipid content (% of total phospholipid)	Drug concentration (μM) causing 25% inhibition of Phospholipase A_1*			Phospholipase A_2 + β-lysophospholipase†		
	PI	PS	PA	PI	PS	PA
18	17 ± 4	44 ± 5	139 ± 16	25 ± 5	38 ± 2	50 ± 4
27	28 ± 5	150 ± 11	310 ± 50	50 ± 2	70 ± 5	200 ± 22
36	64 ± 8	375 ± 44	>300	64 ± 9	89 ± 9	278 ± 25
54	128 ± 17	>375	>300	125 ± 11	222 ± 16	>278

* Release of labelled lysophosphatidylcholine.
† Release of labelled fatty acid.
Each value represents the mean of three determinations ± SD.

Table 2. Effect of gentamicin (G), amikacin (A) and streptomycin (S) on the degradation of labelled phosphatidylcholine (1-palmitoyl, 2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) in function of the nature of the acidic phospholipid included in liposomes (18% of total phospholipids)

Acidic phospholipid included in liposomes	Drug concentration (μM) causing 25% inhibition of Phospholipase A_1*			Phospholipase A_2 + β-lysophospholipase†		
	G	A	S	G	A	S
Phosphatidylinositol	17 ± 4	89 ± 10	146 ± 16	25 ± 5	44 ± 5	97 ± 9
Phosphatidylserine	44 ± 5	150 ± 16	313 ± 32	38 ± 2	106 ± 12	201 ± 18
Phosphatidic acid	139 ± 16	694 ± 81	819 ± 64	50 ± 4	222 ± 20	736 ± 82

* Release of labelled lysophosphatidylcholine.
† Release of labelled fatty acid.
Each value represents the mean of three determinations ± SD.

Table 3. Binding parameters of gentamicin to negatively-charged liposomes containing phosphatidic acid (PA), phosphatidylserine (PS) or phosphatidylinositol (PI) as determined by equilibrium dialysis

Negatively-charged phospholipid			
Content (% of total lipid)	Nature	B_{max}* (nmol gentamicin/nmol acidic phospholipid)	K_d* (μM)
9	PA	0.124 ± 0.008	18.71 ± 3.59
	PS	0.176 ± 0.015	23.58 ± 4.83
	PI	0.125 ± 0.018	47.24 ± 13.36
18	PA	0.262 ± 0.020	13.35 ± 3.10
	PS	0.229 ± 0.015	12.66 ± 2.28
	PI	0.302 ± 0.035	14.63 ± 4.81
27	PA	0.369 ± 0.018	9.60 ± 6.51
	PS	0.269 ± 0.038	15.03 ± 6.51
	PI	0.325 ± 0.029	14.92 ± 3.99
36	PA	0.451 ± 0.154	12.34 ± 5.92
	PS	0.464 ± 0.026	23.51 ± 3.10
	PI	0.347 ± 0.011	7.64 ± 0.93
45	PA	0.431 ± 0.031	23.24 ± 4.24
	PS	0.403 ± 0.042	9.42 ± 4.04
	PI	0.290 ± 0.046	37.26 ± 14.40
54	PA	0.400 ± 0.010	4.00 ± 0.48
	PS	0.340 ± 0.034	3.63 ± 3.99
	PI	0.306 ± 0.027	17.11 ± 4.69

* The parameters were calculated from the direct plots of bound (B) vs free (F) gentamicin concentrations to which an hyperbolic function $B = B_{max}.F/(K_d + F)$ was adjusted by the Taylor's development using the Gauss–Newton method [28, 29]. The resolution of system of normal equations gives the values of K_d (dissociation constant) and B_{max} (maximum capacity of binding) and the matrix of variance–covariance gives the deviation of these parameters.

nanomole of acidic phospholipid increased with the content of the membrane in the corresponding lipid, up to a plateau value of approx. 0.300 nmol of gentamicin per nanomol of acidic phospholipid. No marked, systematic difference among lipids, however, was observed. K_d values ranged between approx. 3–47 μM, with no systematic difference with respect to the nature or the relative content in acidic phospholipid.

Partition studies between two, non-miscible aqueous two phases (polyethyleneglycol/dextran-phosphate) showed that an increase of the proportion of acidic phospholipid included in liposomes resulted in a correspondingly greater accumulation of the vesicles in the dextran-phosphate (bottom) phase, consistent with an increase of their net surface charge [31]. The technique, however, does not allow to directly compare liposomes with different negatively-charged phospholipids in this respect.

The influence of the liposome composition, with respect to the nature and the relative content in acidic phospholipid, on their average size was examined by light scattering spectroscopy. The results so obtained varied around 100 nm, whatever the nature of acidic phospholipid included in the bilayer as long as the content of negatively-charged lipid was less than 27% of total phospholipid. Values obtained when more than 27% of acidic phospholipid was present in the bilayer were, however, variable. The technique used, however, is very sensitive to the minor aggregations which were sometimes observed with these liposomes.

DISCUSSION

In vivo and *in vitro* studies have shown that aminoglycoside antibiotics impair the lysosomal catabolism of phospholipids, causing the intralysosomal accumulation of undegraded polar lipids ('myeloid bodies'), by decreasing the activities of phospholipases (reviewed in Ref. 11). Previous *in vitro* studies using liposomes containing phosphatidylinositol have shown the importance of the binding of aminoglycosides to the bilayer in this respect [6, 18, 20], and its relevance to aminoglycoside toxicity towards kidney or other cells or tissues has been discussed [10, 32]. Most natural membranes, however, also contain other negatively-charged phospholipids such as phosphatidylserine or phosphatidic acid. Phosphatidylserine is a major acidic phospholipid of membranes including the pericellular and lysosomal ones [33], and binding of gentamicin to this lipid has been demonstrated *in vitro* [34, 35]. The membrane content of phosphatidic acid is usually very low but, in many cells and tissues, there is initially an extensive net conversion of phosphatidylinositol to phosphatidate following stimulation to secretion [36–39]. The concentrations of drugs used, the pH at which the experiments were performed, and the type of liposomes used are relevant to the situation prevailing *in vivo* in cells developing typical lysosomal alterations upon aminoglycoside treatment. Thus, aminoglycosides accumulate in the lysosome of proximal tubular cells, or of cultured cells, and these organelles display an acid pH, probably equal to or

lower than 5.5 *in vivo* [40]. In lysosomes, aminoglycosides reach millimolar concentration, even after treatment with, or exposure to, moderate amounts of these antibiotics [5, 8, 41]. Membranes and myeloid bodies isolated from the kidney and urine of aminoglycoside-treated animals and humans show an enrichment in phosphatidylinositol and phosphatidylserine [42–44], which remain within the range investigated here [45]. It is noteworthy that the membrane content in total acidic phospholipids is closely regulated [46, 47], and that mutant cells deficient in the synthesis of phosphatidylinositol contain a correspondingly increased amount of phosphatidylglycerol. In the present models, only one acidic phospholipid was introduced in each type of liposome studied for the sake of simplicity, but a future report will deal with the effect of the simultaneous presence of several acidic phospholipids on phospholipases activity. We have also limited ourselves to examine the catabolism of phosphatidylcholine by phospholipases A_1 and A_2. This phospholipid, however, is a major constituent of all cellular membranes [21], is primarily degraded by phospholipases A_1 and A_2, and is being accumulated to large extents in renal cortex or cultured cells upon aminoglycoside treatment [41–44]. In addition phosphatidylethanolamine, the second other major zwitterionic phospholipid of most membranes, is also degraded by lysosomal phospholipases A_1 and A_2 [48].

In the first part of our study, we observed that phosphatidylserine and phosphatidic acid are as or more effective, than phosphatidylinositol, at increasing the digestive capacities of lysosomal extracts towards phosphatidylcholine. Assuming that the release of ^{14}C-labelled lysophosphatidylcholine and of ^{14}C-labelled fatty acid observed in our experiments is entirely accountable for by the activity of phospholipase A_1, and by the combined activities of phospholipase A_2 and β-lysophospholipase, respectively, as demonstrated earlier for phosphatidylinositol-containing liposomes [6], our results indicate that these enzymes are, generally speaking, highly dependent upon the presence of negative charges in the bilayer in which their substrate is included, as suggested earlier [20]. We, however, have no simple explanation concerning the marked, quantitative differences observed among the negatively-charged phosphoglycerides studied. No important variation in liposome size was observed. Differential distribution of phosphatidic acid across the membrane compared to phosphatidylserine or phosphatidylinositol, as reported for natural membranes [49], is unlikely in view of the lack of constant differences in the binding parameters of gentamicin towards liposomes containing each of the three acidic phospholipids. The activity of lysosomal phospholipases measured on neutral liposomes made of synthetic phosphatidylcholine only is maximal at the temperature of lipid phase transition [50]. It could therefore be suggested that the acidic phospholipids would influence *indirectly* the enzymatic activity of phospholipases, by modifying this temperature [51, 52]. We did not, however, observe any marked change of phospholipases activity over a 15 to 50° range using liposomes of similar composition and negative charge as those used here (unpublished data), and those liposomes are indeed not expected to show a definite, sharp phase transition upon temperature increase in that range. In a study of the hydrolysis of synthetic phosphoglycerides by phospholipase A_2, it was observed [53] that small substituents on the phosphate made better substrates than large apolar substituents, as if enzyme anchored to the interface could more easily interact with the corresponding monomer phospholipid. The same, may be true for the binding of the lysosomal phospholipases to the acidic phospholipids, taking into account the effective size of the hydrated phospholipid headgroups in the bilayer [54]. Alternatively, the isothermal lateral phase separation of the acidic phospholipids could be different, resulting in distinct organization of the negative charges at the surface of the bilayer, and, therefore, in differences in enzyme binding. Whatever the explanation of the differential effect of the negatively-charged phospholipids, on lysosomal phospholipases, it is interesting to note that the activity of other enzymes involved in polar lipid catabolism, such as glucosylceramidase, is also a function of the presence of phosphatidic acid or phosphatidylserine (A.M. Vaccaro, personal communication). Moreover, membrane-proteins such as Na^+/K^+ ATPase [55] are also influenced by negatively-charged lipid.

The results obtained in the second part of our study confirm and extend our previous observations concerning the interactions of aminoglycosides with acidic phospholipids and the inhibitory potency of these drugs towards lysosomal phospholipases. First, they show that the amount of gentamicin that can bind to liposomes per mole of acidic phospholipid increases with the percentage of this negatively-charged phospholipid, as found earlier for phosphatidylinositol-containing liposomes [19]. This, however, is probably more related to the closeness of the negative charges—which make them more available to the drug molecule—rather than to a true cooperative process, since the dissociation constants (K_d) are not significantly influenced by the percentage of acidic phospholipid in the bilayer. These dissociation constants are similar from one phospholipid to another, and close to those obtained by Kubo *et al.* [56] for liposomes made only of phosphatidylcholine (60%) and phosphatidylinositol, phosphatidylserine or phosphatidylglycerol (40%). The present results also confirm and extend our previous observations that amikacin, and still more streptomycin, are much less inhibitory than gentamicin towards lysosomal phospholipases *in vitro* [18]. This observation is probably critical in explaining the lesser nephrotoxicity of these agents compared to gentamicin (see review in Refs 10 and 11). Moreover, the three aminoglycosides tested were found less inhibitory towards phospholipases activity measured on phosphatidic acid- or phosphatidylserine-containing liposomes than was previously observed with phosphatidylinositol even though equilibrium dialysis experiments failed to demonstrate a significant difference in binding parameters of the drug towards each of these liposome populations. Finally, the constant observation that the inhibition of phospholipases by aminoglycosides is inversely pro-

portional to the bilayer content in acidic phospholipids supports our hypothesis concerning the importance of surface charge neutralization in this process. In this respect, our results may be compared to those obtained with Adriamycin®, which inactivates cytochrome *c*-oxidase by binding to cardiolipin, a negatively-charged phospholipid essential for its activity [57]. The results of the dialysis experiments, however, clearly demonstrate that gentamicin binding to the bilayer at equilibrium is not the only parameter governing the drug-induced phospholipase inhibition, when comparing liposomes with different acidic phospholipids. This conclusion was already suggested by the study of streptomycin derivatives such as streptomycylamine, which tightly binds to negatively-charged liposomes but are poorly inhibitory [30]. The results of a conformational analysis examining the interactions of gentamicin and amikacin with the three types of negatively-charged phospholipids, and reported in a companion paper [23], may explain these discrepancies between binding and inhibitory potency which could not clearly be revealed from studies using only one type of negatively-charged liposomes [18]. It must be stressed, however, that we have not measured the actual surface potentials of liposomes containing phosphatidylinositol, phosphatidylserine or phosphatidic acid, and their change upon addition of aminoglycosides under our experimental conditions. Binding of the same amount of a given aminoglycoside (at equilibrium) to different types of negatively-charged liposomes does not, indeed, necessarily imply that the original surface potentials and the changes caused by addition of the different aminoglycosides studied are the same.

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