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BIOCHEMICAL MECHANISM OF AMINOGLYCOSIDE-INDUCED INHIBITION OF PHOSPHATIDYLCHOLINE HYDROLYSIS BY LYSOSOMAL PHOSPHOLIPASES

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Abstract—Aminoglycosides such as gentamicin are hydrophilic, polycationic drugs which bind to negatively-charged phospholipid bilayers, inhibit the activities of the lysosomal enzymes involved in the degradation of the major phospholipids and cause, in kidney *in vivo* or in cultured cells, a lysosomal phospholipidosis. In the present study, we show that the hydrolysis of phosphatidylcholine included in liposomes by lysosomal extracts at pH 5.4 *in vitro* is critically dependent on the negative charges carried by the bilayer. This hydrolysis, which is predominantly carried on by phospholipases A1 and A2, markedly increases when the phosphatidylinositol content is raised from 10 to 30% of the total phospholipids, i.e. in a range found in natural membranes. Addition of gentamicin decreases the activity of these enzymes in a non-competitive fashion, but the effect is inversely proportional to the amount of phosphatidylinositol present in the bilayer. Gentamicin and bis(beta-diethylaminoethylether)hexestrol (DEH), a cationic amphiphile which also binds to phospholipid bilayers, are equipotent inhibitors when added to negatively-charged liposomes at equinormal concentrations. Although direct aminoglycoside–enzyme interactions cannot be excluded, these results strongly suggest that gentamicin impairs the activities of the lysosomal phospholipases towards phosphatidylcholine by decreasing the available negative charges required for optimal activity.

Aminoglycoside antibiotics remain essential in clinical practice for the treatment of infections caused by Gram (−) bacteria, because of their excellent chemotherapeutic properties [1, 2]. The limiting factor in their use remains their oto- and nephrotoxicity and these adverse effects have deterred the clinical development of several new, therapeutically-promising compounds [3]. With respect to nephrotoxicity, aminoglycosides have been shown to concentrate in the lysosomes of kidney proximal tubular cells [4–7], to inhibit the activities of phospholipases A and C, and sphingomyelinase [6, 8–10] and to induce a phospholipidosis, characterized by the formation of myeloid bodies [6, 11]. The latters consist of a complex mixture of phospholipids naturally present in the cell, even though they show a significant enrichment in phosphatidylinositol [12, 13]. Certain evidence (reviewed in Ref. 14) strongly suggests that the nephrotoxicity of aminoglycosides is related to the extent of this phospholipidosis. Its mechanism, however, remains partly uncovered. Inhibition of lysosomal phospholipases by aminoglycosides can be reproduced *in vitro* [8, 9], and it has been proposed that it results from the formation of complexes between these drugs and the phospholipids, when these are organized as bilayers (liposomes) containing negatively-charged phospholipids [15, 16]. In this paper, we examine the influence of the negative charge carried by bilayers on the degrading activities of lysosomal enzymes towards the major phospholipid, phosphatidylcholine, and the effect of gentamicin, taken as a typical aminoglycoside, on these enzyme activities.

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MATERIALS AND METHODS

Preparation of liposomes. Unless stated otherwise, sonicated liposomes were prepared from cholesterol, egg yolk phosphatidylcholine, bovine brain sphingomyelin and wheat germ phosphatidylinositol. The phospholipid : cholesterol molar ratio was set at 2 : 1, and the phosphatidylcholine content was kept constant at 4 mol per 11 mol of phospholipid throughout. The negative charge of the liposomes was varied by adding increasing amounts of phosphatidylinositol (from 0 up to 6 mol), whereas the sphingomyelin content was correspondingly decreased from 7 mol (for neutral liposomes) to 1 mol per 11 mol of phospholipids (negatively-charged liposomes). The liposomes contained 140 mCi of labelled phosphatidylcholine (1-palmitoyl, 2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) per mol of phosphatidylcholine. They were routinely prepared in acetate buffer at pH 5.4 as described earlier [8] except that the concentration of the buffer was 40 mM instead of 4 mM in order to keep a constant pH in spite of widely different phosphatidylinositol contents. The final lipid concentration was 10 g/l. Liposomes were stored under nitrogen and used within a week.

Enzyme source. The enzyme source was a soluble

fraction of purified liver lysosomes, isolated from rats treated with Triton WR 1339 as previously described [17, 18]. The main biochemical characteristics of this fraction with respect to purity, phospholipase activity and phospholipid content have been summarized elsewhere [15] and are consistent with other authors' preparations [19].

Measurement of labelled phosphatidylcholine breakdown. The activity of the lysosomal phospholipase A1 (phosphatidate 1-acylhydrolase, EC 3.1.1.32) and the sum of phospholipase A2 (phosphatidate 2-acylhydrolase, EC 3.1.1.4) and betalysophospholipase (lysolecithin 2-acylhydrolase, EC 3.1.1.-) activities towards 1-palmitoyl, 2-[1-^{14}C]oleoyl phosphatidylcholine included in the liposomes were determined by measuring the release of ^{14}C-betalysophosphatidylcholine (2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) and [1-^{14}C]oleic acid respectively, as described previously [8, 15]. In brief, oleic acid, lysophosphatidylcholine and unhydrolyzed phosphatidylcholine were separated by ascending thin-layer chromatography and the amount of radioactivity associated with each of these constituents expressed in percent of the radioactivity recovered. As shown earlier [10], lysosomal phospholipases A1 and A2 are not active against phosphatidylinositol under these assay conditions. Conversely, phospholipase C is active against phosphatidylinositol [10], but not against phosphatidylcholine [15]. Unless stated otherwise, drugs and liposomes were preincubated at 37° for 1 hr under nitrogen in 40 mM acetate buffer at pH 5.4. The reaction was started by addition of the enzyme preparation and carried out for 30 min. Phosphatidylcholine concentration in the assay mixture was 1.93 mM. The drug solutions and the enzyme preparation were diluted in the incubation buffer in order to avoid pH changes of the incubation mixture. All measurements in a given experiment were made in triplicate.

Calculations. For the kinetic studies, the fitting of the experimental points to the Michaelis–Menten

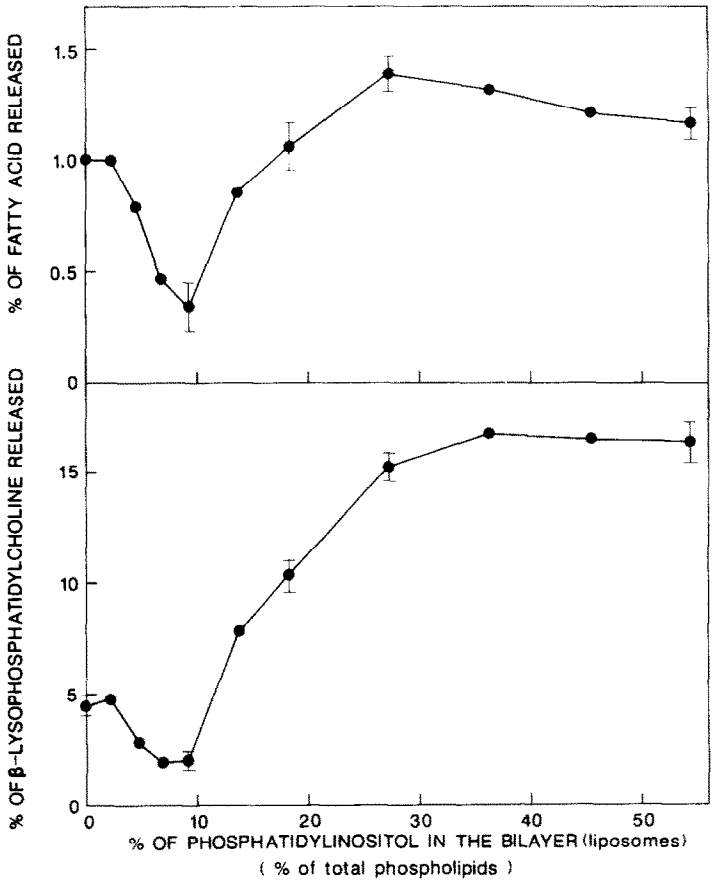

Fig. 1. Degradation of 1-palmitoyl-2-[1-^{14}C]oleoyl phosphatidylcholine included in liposomes as a function of their phosphatidylinositol content (in percent of the total phospholipids) in the presence of lysosomal extracts at pH 5.4. The amounts of labelled compound released are shown on the ordinates and expressed as the percentage of the total radioactivity present in the assay mixture. The upper part of the diagram shows the release of labelled oleoic acid (activities of phospholipase A2 and betalysophospholipase); the lower part shows the release of labelled lysophosphatidylcholine (2-oleoyl-*sn*-glycero-3-phosphocholine) (activity of phospholipase A1). Each symbol with a vertical bar (±standard deviation) refers to the mean of at least three separate experiments; symbols without bar refer either to a single experiment or the mean of two experiments.

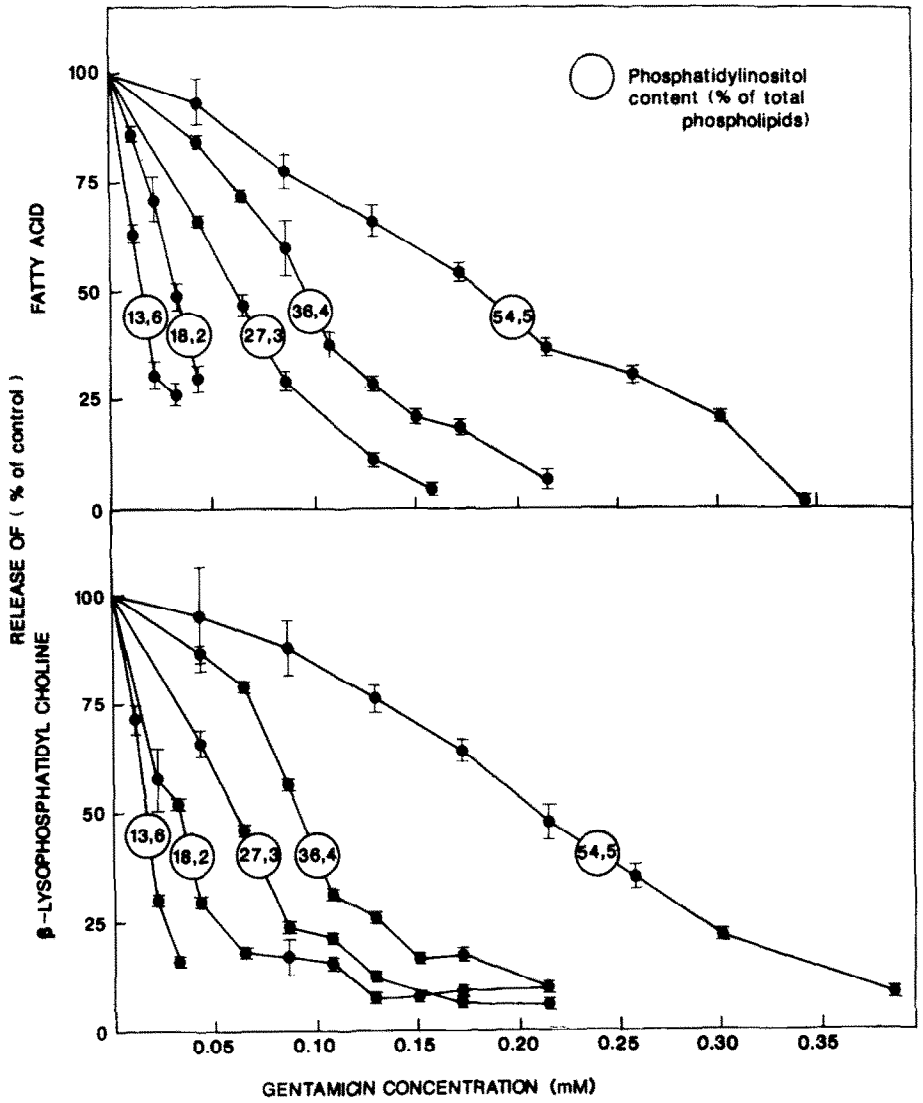

Fig. 2. Effect of gentamicin on the degradation of labelled phosphatidylcholine included in liposomes containing increasing amounts of phosphatidylinositol in the presence of lysosomal extracts at pH 5.4. The abcissa shows the actual concentrations of antibiotic in the final assay mixture. The figures in the circles superimposed to each curve show the phosphatidylinositol content of the liposomes in percent of the total phospholipids. The ordinate gives the amount of reaction product as the percentage of that measured in parallel experiments without antibiotic. The upper part of the diagram shows the activities of phospholipase A2 and beta-lysophospholipase, the lower part the activity of phospholipase A1. Each value (±SD) represents the mean of 3 to 9 determinations.

equation was made by non-linear regression using the Gauss–Newton method [20]. Since our measurements were relative (i.e. we measured percentages of hydrolysis), data were weighed by their reciprocal value ($1/y$). Statistical comparison of the equation parameters (K_m; V_{max}), was made by the Student's t-test (considering K_m and V_{max} independently) and by the method of indifference regions [21] in which both K_m and V_{max} are first calculated by the covariance matrix method and are thereafter systematically varied to cover the 95% confidence region of the combined values of K_m and V_{max}.

Materials. Gentamicin (sulphate salt) was supplied by Schering Corporation (Kenilworth, NJ) as "Gentamicin reagent solution" for *in vitro* and investigational use. Like other commercial preparations of gentamicin, it consisted of a mixture of 3 main components, gentamicin C1, C1a and C2 in a molar ratio of approx. 30:30:40. The inhibitory potency of the isolated components towards lysosomal phospholipases *in vitro* does not, however, significantly differ from that of the commercial mixture [15]. Bis(beta-diethylaminoethylether)hexestrol (DEH) was a gift from the Upjohn Company (Kalamazoo, MI). The natural glycerophospholipids (egg yolk phosphatidylcholine, wheat germ phosphatidylinositol) were purchased from Lipid Products (Redhill, U.K.) as grade 1 products. Bovine

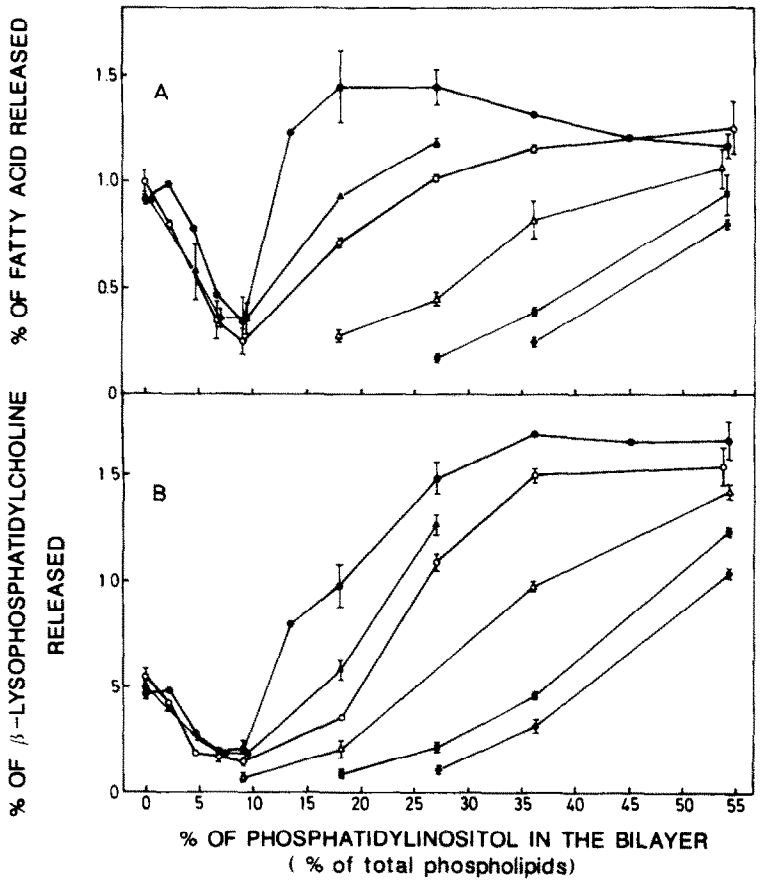

Fig. 3. Influence of the simultaneous variation of the phosphatidylinositol content of the liposomes and the gentamicin concentration on the activity of the lysosomal phospholipase A1 (bottom) and of phospholipase A2 + betalysophospholipase (top) at pH 5.4. The experiments were conducted as in Figs 1 and 2, and the data represented as in Fig. 1. Each curve links all experimental data obtained at a similar gentamicin concentration (●, no gentamicin; ▲, 0.022 mM; ○, 0.040 mM; △; 0.090 mM; ■, 0.130 mM and ◆, 0.170 mM) in the assay mixture.

brain sphingomyelin and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO) and radiolabelled phosphatidylcholine from Amersham International plc (Amersham, U.K.). Other reagents were obtained from E. Merck, AG (Darmstadt, F.R.G.) and were of analytical grade.

RESULTS

Figure 1 shows the release of labelled lysophosphatidylcholine and labelled fatty acid from phosphatidylcholine present in liposomes containing increasing amounts of phosphatidylinositol upon incubation in the presence of lysosomal extracts at pH 5.4. Whereas the liberation of labelled lysophosphatidylcholine is entirely accountable for by the activity of phospholipase A1, about two-thirds of the free labelled fatty acid liberated in our conditions arises from the activity of phospholipase A2 and one-third from the activity of betalysophospholipase [8]. These activities markedly increased when the phosphatidylinositol content was varied from 10 to 30% of total phospholipids, above which a plateau was reached. Interestingly enough, the activities measured at a phosphatidylinositol content of approx. 10% were minimal, and rose again when the phosphatidylinositol content was further decreased. On neutral liposomes, activities amounted to approx. 25 and 70% of the maximal activities observed with negatively-charged liposomes for the release of lysophosphatidylcholine and fatty acid respectively. In order to check that the lower activities found in neutral liposomes were not caused by the large amount of sphingomyelin (added to replace phosphatidylinositol without modifying the phospholipid:cholesterol ratio), neutral liposomes were prepared with phosphatidylcholine and cholesterol only. No significant difference in activity was seen as compared to neutral liposomes prepared with sphingomyelin, taking into account the larger phosphatidylcholine concentration. Results similar to those shown in Fig. 1 were obtained if phosphatidylserine or phosphatidic acid were included in the liposomes instead of phosphatidylinositol (data not shown). The enzyme activities were therefore considered dependent on the negative charges displayed by the bilayer, and all further studies were carried on with liposomes containing phosphatidylinositol only as a negatively-charged phospholipid.

Previous work from our laboratory had shown that

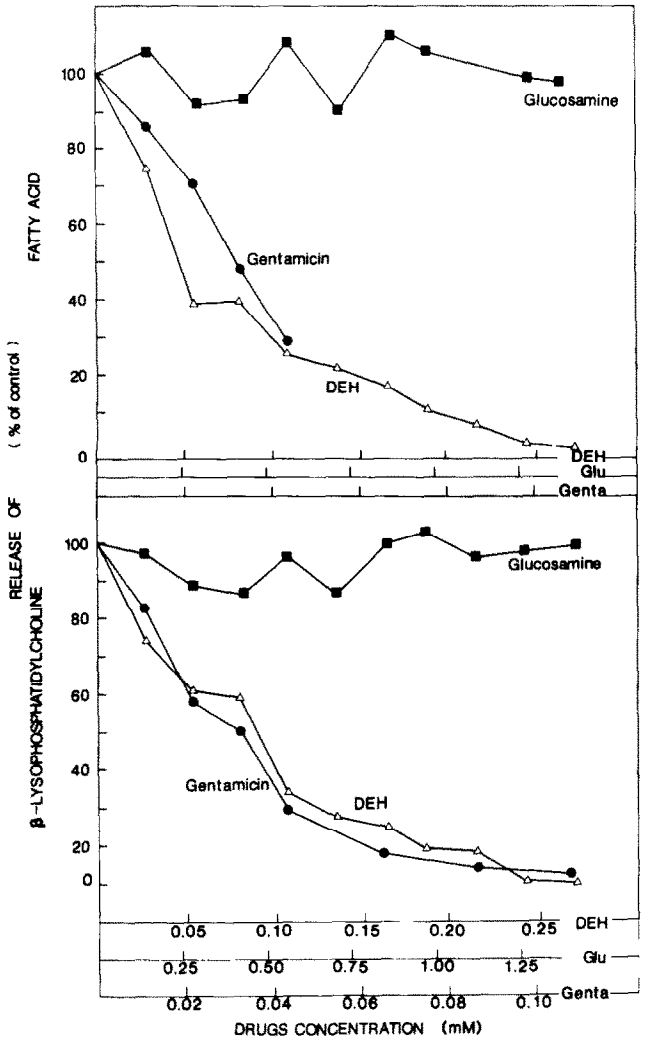

Fig. 4. Comparative inhibition of the activities of phospholipase A1 (lower part), phospholipase A2 and beta-lysophospholipase (upper part) by gentamicin, bis(beta-diethylaminoethylether) hexestrol (DEH) and glucosamine. The experiment was performed with liposomes containing 18.2% of phosphatidylinositol (in percent of total phospholipids) in the conditions described in Fig. 2. The abcissas show the final molar concentrations of gentamicin (Genta), DEH and glucosamine (Glu) in the assay mixtures. In order to facilitate the comparison, the scales have been adjusted so that each point on the abcissa corresponds to an *equinormal* concentration of the three compounds studied.

gentamicin inhibits, in a dose-dependent fashion, the activities of lysosomal phospholipases towards phosphatidylcholine included in negatively-charged liposomes [8], whereas it is without effect when assays are performed with neutral liposomes [15]. In the present study, we therefore systematically examined the influence of the negative charge on the inhibitory effect of gentamicin. Figure 2 shows that this inhibition is indeed dependent on the drug concentration but is also inversely proportional to the percentage of phosphatidylinositol included in the bilayer when it is varied from 13.6 to 54.5% of total phospholipids. When the results of Figs 1 and 2 are combined, as shown in Fig. 3, it clearly appears that the effect of gentamicin is to shift, in a dose-dependent fashion, the curves expressing the enzyme activities relative to the phosphatidylinositol content when the latter is higher than 10%. Figure 3 also shows that gentamicin exerts no significant effect on neutral liposomes. For liposomes containing less than 10% of phosphatidylinositol, little inhibition was observed and this inhibition was not dose-dependent. Since natural membranes, and especially the lysosomal membrane, contain more than 10% of negatively-charged phospholipids [22, 23], neutral liposomes were not further investigated, and subsequent studies were performed with liposomes containing a fixed amount of phosphatidylinositol of 18.2%.

Gentamicin carries 5 cationic groups and tightly binds to negatively-charged phospholipids at acid pH [8, 24], mostly through electrostatic interactions [16]. We therefore compared its effect to that of an amphiphilic cationic drug, bis(beta-diethylamino-

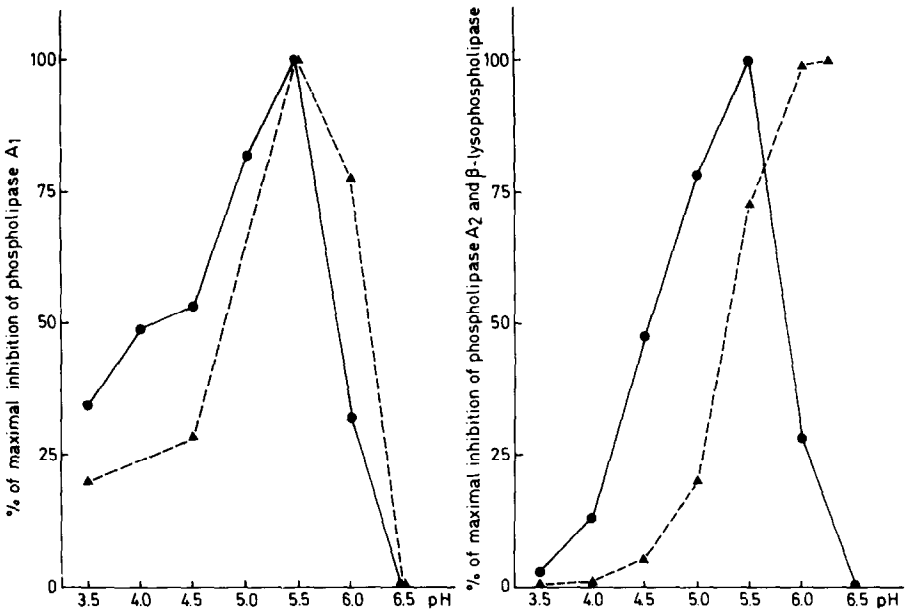

Fig. 5. Influence of pH on inhibitory potency of gentamicin (●, ——, 0.02 mM) or DEH (▲, – – –, 0.05 mM) on the activities of phospholipase A2 + beta-lysophospholipase (right panel) and phospholipase A1 (left panel) towards liposomes containing 18.2% of phosphatidylinositol (in percent of total phospholipids). The liposomes were prepared in 40 mM McIlvaine's citric acid/Na_2HPO_4 buffer adjusted at pH values ranging from 3.5 to 6.5 and all subsequent dilutions were performed in the corresponding buffer. For each pH value, we calculated the inhibition (I) as $I = 1 - A/B$ where A is the activity in the presence of drug and B the activity found with control liposomes. In order to normalize the data for both phospholipases A1 and A2, and gentamicin and DEH, I is then expressed for each drug in percent of the maximal inhibition observed over the pH range studied and this value is used in the ordinate. Data are from a single experiment, but each value is the mean of 3 determinations.

ethylether)hexestrol (DEH). This compound carries only 2 cationic groups but also inhibits lysosomal phospholipases A [25]. Because of its structure, it also binds to phospholipid bilayers, but largely by hydrophobic interactions (Mingeot-Leclercq *et al.*, in preparation). Figure 4 shows that gentamicin and DEH are almost equally potent in inhibiting phosphatidylcholine hydrolysis when both compounds are added to the liposome preparations at equinormal concentrations. Conversely, D-2-glucosamine, a monoaminated sugar which does not bind to liposomes under these experimental conditions (data not shown), did not inhibit phospholipase activities up to molar concentrations 50-fold higher than those necessary to obtain a 50% inhibition with gentamicin.

Since the binding of gentamicin to negatively-charged liposomes was shown to be pH-dependent [8], we investigated the influence of pH on gentamicin-induced inhibition of the lysosomal phospholipases, and compared it to that caused by DEH. For these experiments, liposomes were prepared in 40 mM McIlvaine's buffer (citric acid/Na_2HPO_4) as used by Kunze *et al.* [19]. Figure 5 shows that inhibition of phospholipase A1 was maximal for both compounds at pH 5.5. With respect to the liberation of labelled fatty acid, gentamicin was also most inhibitory at pH 5.5, whereas the inhibition caused by DEH markedly rose from pH 3.5 to 6.5.

Figure 6 shows the substrate-dependence of phospholipase A1 activity towards phosphatidylcholine in the absence and in the presence of 0.02 mM gentamicin or 0.05 mM DEH, i.e. at concentrations of inhibitors causing approx. a 40% decrease of activity. Only substrate concentrations below 1.7 mM were explored, since preparing liposomes at significantly higher lipid concentration than used here (10 g/l) could have resulted in changes of critical biophysical properties such as vesicle size or ratio of accessible to inaccessible phospholipids. The kinetic parameters, calculated from the fitting of the experimental points to the Michaelis–Menten equation by non-linear regression, and their confidence intervals are shown in Table 1. These parameters were used to draw the curves and lines showed in Figs 6(A) and (B). The fitting of the experimental points to the Michaelis–Menten equation was satisfactory for the uninhibited enzyme. In presence of DEH, V_{max} was significantly decreased whereas K_m remained essentially unchanged. For gentamicin, a similar conclusion was reached, although examination of the data in inverse coordinates (Fig. 6B) revealed that the parameters of Table 1 systematically underestimated the rate of the reaction at the lower substrate concentrations, suggesting that these parameters perhaps could be different at high and low gentamicin/substrate ratios.

DISCUSSION

Upon accumulation in cultured cells, or in kidney *in vivo*, aminoglycosides induce an increase in the

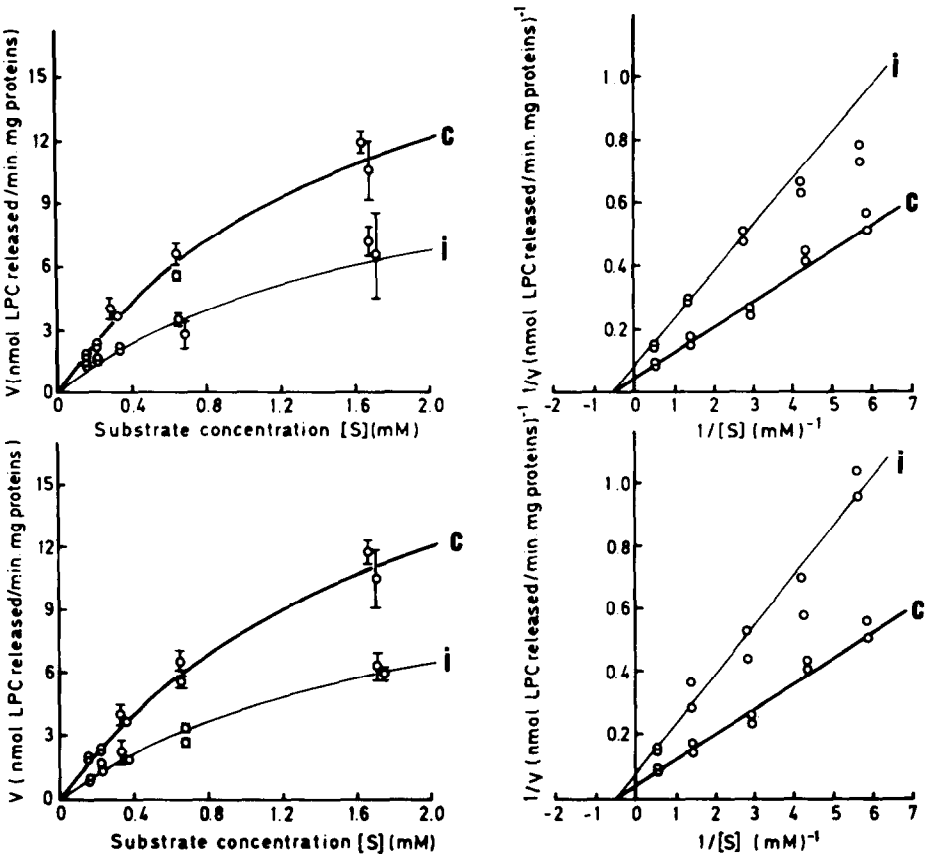

Fig. 6. Influence of the substrate concentration on the activity of phospholipase A1 in the absence (c) or in the presence (i) of 0.02 mM gentamicin (upper diagrams) or 0.05 mM DEH (lower diagrams). The left diagrams show the data in direct coordinates (rate of reaction (v) vs substrate concentration S), and the right diagrams in inverse coordinates ($1/v$; $1/S$). Two independent experiments were performed. Symbols refer to the mean values of 3 determinations (±SD; left diagram) performed at each substrate concentration for each experiment. The curves (left) and lines (right) have been drawn based on the mean parameters K_m and V_{max}, determined by fitting all experimental points from the two experiments to the Michaelis–Menten equation by non-linear regression, and shown in Table 1.

cellular concentration of all major phospholipids [12, 13, 26, 27]. This metabolic disturbance has been ascribed to an inhibitory effect of aminoglycosides on the enzymes responsible for the lysosomal catabolism of these constituents, namely phospholipase A1, A2, C, phosphatidylinositol phospholipase C and sphingomyelinase [8–10, 26]. The present study extends our previous *in vitro* analyses [8, 15, 16] of the inhibitory effect exerted by gentamicin on the hydrolysis of phosphatidylcholine included in arti-

Table 1. Kinetic parameters of phospholipase A1

Inhibitor	K_m (mM)	V_{max} (nmol × min^{-1} × mg^{-1} protein)
None (control)	1.77 ± 0.21*	22.8 ± 1.9
	(1.23–2.31)†	(17.8–27.8)
Gentamicin 0.02 mM	1.87 ± 0.50	13.1 ± 2.4‡
	(0.60–3.14)	(6.7–19.4)
DEH 0.05 mM	1.90 ± 0.33	12.9 ± 1.6‡
	(1.08–2.75)	(8.8–16.9)

The kinetic parameters were calculated by fitting all experimental points of 2 independent experiments—the mean values (±SD; N = 3) for each of them being shown in Fig. 6—to the Michaelis–Menten equation, using non-linear regression.

* ±Standard deviation, the values of K_m and V_{max} being analyzed separately.

† 95% confidence interval calculated by the method of indifference regions in which both K_m and V_{max} are varied together [21].

‡ $P < 0.01$ by Student t-test, as compared to V_{max} of control.

ficial bilayers (liposomes) by extracts of purified lysosomes, a process which is predominantly carried out by phospholipases A1 and A2. Liver lysosomes were used for this study since these are more easily purified than kidney lysosomes and have proved to be a useful model in the comparative study of several clinically-used and experimental aminoglycosides [8, 15, 28–30].

Hydrolysis of phosphatidylcholine by lysosomal extracts appears critically dependent on the negative charge carried by the liposomes. This result is in agreement with those obtained with purified lysosomal phospholipase A1 towards phosphatidylcholine and phosphatidylethanolamine [31]. The range of phosphatidylinositol content over which we found the activities of lysosomal phospholipases to vary *in vitro* is physiologically relevant, since most membranes of eucaryotic cells, including the lysosomal membrane, show a content in negatively-charged phospholipids between 10 and 30% [22, 23, 32].

If the density of negative charges at the surface of the bilayer is an essential factor modulating the activity of lysosomal phospholipases towards phosphatidylcholine, we may postulate that gentamicin, which binds to acidic phospholipids [8, 16, 33], decreases the amount of negative charges available to the enzymes, and thus impairs their activity. This would explain why these activities can remain unaffected in the presence of gentamicin if the amount of phosphatidylinositol included in the bilayer is increased in such a way that the original negative charge is kept. This explanation must remain qualitative, however, since we have not obtained direct measurements of the number of accessible negative charges at the surface of the liposomes at increasing phosphatidylinositol contents and/or upon addition of gentamicin. Yet, gentamicin decreases the electrophoretic mobility of negatively-charged liposomes [24], and it has been shown that the activity of phospholipase A1 towards phospholipid vesicles varies with this electrophoretic mobility [31].

The results obtained with DEH provide further evidence for the role played by the decrease of the number of negative charges in phospholipase inhibition. Both drugs inhibit phospholipase activities to a similar extent at the same positive charges/phospholipids ratios even though their mode of insertion into the bilayer and the type of interactions established with the phospholipids are largely different [16, 34; Mingeot-Leclercq *et al.*, in preparation). This result does not necessarily contradict those showing a correlation between the hydrophobicity of cationic amphiphilic drugs carrying a similar number of cationic charges and their inhibitory potency towards phospholipase [35], but they indicate that hydrophobicity is not the only parameter to consider. We, ourselves, showed that hydrophobic derivatives of aminoglycosides, such as benzyl or butylstreptomycylamine, are less inhibitory than gentamicin on a molar basis, partly because they carry less cationic charges [34].

Assuming that gentamicin and DEH both inhibit phospholipase A1 by decreasing the number of available negative charges, the results of the kinetic analyses would suggest that the affinity of the enzyme for phosphatidylcholine is not modified under these conditions, but that its reaction rate is slowed down. The data would, however, be also consistent with the formation of enzyme–drug complexes, as was shown to occur with other cationic drugs [36]. Yet, no evidence of a direct interaction between aminoglycosides with phospholipase A1 has been presented so far, but definitive studies in this direction will probably require availability of a highly purified enzyme.

The data shown in this paper may explain why lysosomal breakdown of phosphatidylcholine is severely impaired in kidneys of animals treated with aminoglycosides or in cultured cells incubated in the presence of these drugs. As discussed in Refs. [6, 7] and [26], the intralysosomal concentration of gentamicin in proximal tubular cells or in cultured cells easily reaches millimolar concentrations, even after treatment with, or exposure to, moderate amounts of this antibiotic. Moreover, even though membranes and myeloid bodies isolated from the kidneys of aminoglycoside-treated animals show an enrichment in phosphatidylinositol [12, 13, 27], the concentration of this phospholipid remains lower than 30% and is thus insufficient to compensate for the huge amounts of gentamicin present. The observation that inhibition of phospholipase A1 is maximal at pH 5.5 for both gentamicin and DEH may also have a biological significance since this is the pH considered to prevail in lysosomes *in vivo* [37, 38]. Between pH 6.5 and pH 5.5, both DEH and gentamicin tend to become more protonated [39] and are therefore expected to bind more tightly to liposomes. We have, however, no simple explanation for the decrease in inhibitory potency seen at a lower pH. For phospholipase A2, a similar mechanism may be involved for gentamicin, but not for DEH. In this case, perhaps, as for chloroquine [19], hydrophobic interactions may become predominant.

In conclusion, our experiments show the importance of the negative charges exhibited by the membranes for the hydrolysis of phosphatidylcholine by lysosomal enzymes and strongly suggest that gentamicin impairs this catabolism by decreasing their density. Pending specific investigations, we may suggest that these conclusions also apply to the other major zwitterionic phospholipid, phosphatidylethanolamine, since both are probably hydrolyzed in lysosomes by the same enzymes [19, 31, 40].

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