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SHORT COMMUNICATIONS

Effect of substrate organization on the activity and on the mechanism of gentamicin-induced inhibition of rat liver lysosomal phospholipase A1

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Abstract—Aminoglycoside antibiotics, such as gentamicin, induce a lysosomal phospholipidosis in the kidney cortex of experimental animals and humans. *In vitro*, gentamicin binds to negatively charged phospholipids, such as phosphatidylinositol, and decreases the activity of lysosomal phospholipases towards a neutral phospholipid (phosphatidylcholine) included in lipid vesicles. The mechanism of such an inhibition was not unequivocally established. On one hand Mingeot-Leclercq *et al.* (*Biochem Pharmacol* **37**: 591–599, 1988) observed that the activity of phospholipase A1 is modulated by the negative charges of the bilayer and that the inhibitory potency of gentamicin is inversely related to the phosphatidylinositol content of the vesicles, and therefore proposed that inhibition is due to charge neutralization. On the other hand, Hostetler and Jellison (*J Pharmacol Exp Ther* **254**: 188–191, 1990) observed that the activity of phospholipase A1 is not modulated by the negative charges of the vesicles and that the inhibitory potency of gentamicin is directly related to the phosphatidylinositol content of the bilayer, and therefore proposed that inhibition is due to substrate depletion. However, the experimental designs of these two models differed in several respects such as the source (liver versus kidney) and nature of the enzyme (native lysosomal extract versus purified delipidated phospholipase A1), and the composition of lipid vesicles (those containing constant amounts of phosphatidylcholine and cholesterol, and inversely varying amounts of phosphatidylinositol and sphingomyelin versus those containing inversely related amounts of phosphatidylcholine and phosphatidylinositol only). In order to assess the nature of the differences between these models, we compared the activity of phospholipase A1 and its inhibition by gentamicin using only one source of enzyme, the rat liver lysosomal extract, and the two types of lipid vesicles as used in the above models. Our results showed that both models are true within the frame work of their respective experimental designs. However, since the composition of the lipid vesicles as well as the nature of the enzyme preparation (whole lysosomal extract) in the "charge neutralization" model is closer to *in vivo* conditions, we suggest that this model may be more relevant to the *in vivo* situation.

Aminoglycoside antibiotics such as gentamicin are nephrotoxic. In both experimental animals and humans, gentamicin causes an early lysosomal phospholipidosis in the proximal tubular cells of the kidney which is likely to result from its accumulation into these organelles after pinocytosis from the luminal fluid (reviewed in Ref. 1). *In vitro*, under conditions of pH(5.4) mimicking those assumed to prevail in lysosomes, gentamicin binds to liposomes containing negatively charged phospholipids and decreases the rate of hydrolysis of phosphatidylcholine included in these vesicles by lysosomal phospholipases [2]. Mingeot-Leclercq *et al.* [3] have explained this effect by showing that phospholipase A1 activity (towards phosphatidylcholine) increases when the bilayer is made negatively charged (e.g. by inclusion of phosphatidylinositol) and have therefore suggested that gentamicin acts by decreasing the amount of surface negative charge causing a non-competitive type of inhibition (*charge neutralization model*). Using an apparently similar approach, Hostetler and Jellison [4], however, did not observe an influence of phosphatidylinositol on phospholipase A1 activity, but nevertheless demonstrated a gentamicin-induced inhibition of this enzyme which could be reversed by increasing the substrate concentration. They therefore ascribed this inhibition to *substrate depletion*, suggesting that gentamicin bound to the negatively charged vesicles would decrease the accessibility of the enzyme to the phosphatidylcholine. However, differences were noted in the experimental conditions used by these two groups with respect to enzyme source and substrate nature and environment. Mingeot-Leclercq *et al.* [3] used a soluble fraction prepared from lysosomes isolated from rat livers (thus containing a mixture of all soluble lysosomal constituents, including phospholipases). The actual substrate was 1-palmitoyl-2-oleoyl phosphatidylcholine presented in liposomes containing, in addition, cholesterol, sphingomyelin and phosphatidylinositol. Hostetler and Jellison [4] used a purified phospholipase A1 prepared from rat kidney and the substrate was presented in vesicles made of dioleoylphosphatidylcholine and phosphatidylinositol only. In order to determine whether the differences between the results obtained with these two models relate to enzyme source or substrate nature and environment, we have examined and report here the activity and gentamicin-induced inhibition of phospholipase A1 from a soluble fraction of rat liver lysosomes towards the two types of substrate preparations.

Materials and Methods

Preparation of substrates. Two types of substrate (henceforth referred to as type 1 and type 2 vesicles) were prepared following the compositions shown in Table 1, and were those used by Mingeot-Leclercq *et al.* [3] and Hostetler and Jellison [4], respectively. The charge of both types of vesicle was varied by increasing the phosphatidylinositol content as described in the original publications [3, 4], i.e. by maintaining a constant level of phosphatidylcholine and manipulating in an inverse way the phosphatidylinositol and sphingomyelin contents of type 1 vesicles, and by a simultaneous but inverse variation of the phosphatidylinositol and phosphatidylcholine contents of type 2 vesicles. In both cases, trace amounts of labelled phosphatidylcholine (1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine) was added at a final concentration of 140 μCi/mmol of phosphatidylcholine.

Enzyme source and assay of enzyme activity. The enzyme

Table 1. Composition of type 1 and type 2 vesicles

Lipid	Relative molar content							
	Type 1				Type 2			
	A*	B	C	D	A	B	C	D
Cholesterol	5.5	5.5	5.5	5.5	None	None	None	None
Phosphatidylcholine†	4.0	4.0	4.0	4.0	11.0	10.5	9.0	7.0
Phosphatidylinositol‡	0.0	0.5	2.0	4.0	0.0	0.5	2.0	4.0
Sphingomyelin§	7.0	6.5	5.0	3.0	None	None	None	None

The composition of type 1 and 2 vesicles relates to those used by Mingeot-Leclercq *et al.* [3] and Hostetler and Jellison [4], respectively. Vesicles were prepared by drying lipids into thin film and then sonicating in 40 mM Na acetate buffer pH 5.4, at a final phospholipid concentration of 10 mM (type 1 vesicles) or 5 mM (type 2 vesicles).

* Letters (A–D) refer to vesicles of increasing negative charge, with phosphatidylinositol content varying from 0 to 36.4% of total phospholipids (see Fig. 1).

† From egg yolk; ‡ from wheat germ; § from bovine brain.

Table 2. Influence of phosphatidylinositol on the activity of phospholipase A1 measured towards phosphatidylcholine in type 1 and type 2 vesicles

Phosphatidylinositol content (% of total phospholipids)	Activity*	
	Type 1	Type 2
0.0 (A)†	4.46 ± 0.70	63.77 ± 5.22§
4.5 (B)	7.34 ± 0.34	65.53 ± 3.91§
18.2 (C)	37.89 ± 4.82‡	63.09 ± 4.07§
36.4 (D)	38.25 ± 4.05‡	54.80 ± 1.21§

* nmol lysophosphatidylcholine released/(min · mg protein).

† Letters (A–D) refer to the compositions described in Table 1.

‡ Significantly higher than values obtained with the corresponding type of vesicle containing no phosphatidylinositol (Student's *t*-test; $P < 0.01$).

§ Significantly higher than the corresponding values for type 1 vesicles (Student's *t*-test; $P < 0.01$).

source was a lysosomal soluble fraction from rat livers prepared as described earlier [2] and enriched 65-fold in phospholipase A1 (phosphatidate 1-acylhydrolase, EC 3.1.1.32) compared to the original homogenate. All incubations were performed in 40 mM Na acetate buffer at pH 5.4 and a typical experiment used 15 μg of lysosomal proteins incubated at 37° for 30 min in a final volume of 80 μL. The final concentration of phosphatidylcholine in the assay mixtures was set at 1.8 mM for type 1 substrate preparations and varied from 1.6 to 2.5 mM for type 2 substrate preparations. All other conditions were as described in Ref. 2.

Results

Table 2 shows that the activity of lysosomal phospholipase A1 towards phosphatidylcholine was directly correlated with the membrane content of phosphatidylinositol in type 1 vesicles, but that phosphatidylinositol had no marked influence on this activity in type 2 vesicles. At any given concentration of phosphatidylinositol, activity towards type 2 vesicles was significantly higher than that towards type 1 vesicles. As shown in Fig. 1, gentamicin exerted no or inconsistent inhibition of phospholipase A1 when tested with neutral vesicles of either type 1 or type 2. Inhibition was modest and incomplete with both types of vesicles containing a low (4.5%) content of phosphatidylinositol. With larger contents of phosphatidylinositol, however, gentamicin exerted quite different effects depending on the type of vesicles. In type 1 vesicles, gentamicin almost completely inhibited the enzyme activity in a dose-dependent fashion but, most conspicuously, the inhibitory potency of the drug in the 0–80% inhibition range was inversely proportional to the phosphatidylinositol content. In type 2 vesicles, inhibition was incomplete; yet the degree of inhibition eventually reached was directly proportional to the phosphatidylinositol content. These results essentially reproduce, with a single source of enzyme, those of Mingeot-Leclercq *et al.* [3] and of Hostetler and Jellison [4].

The differences between the data generated with the two types of vesicles and their relevance to the two modes of inhibition proposed [3, 4] are made more straightforward when the effect of variations in the phosphatidylinositol content and gentamicin concentration are presented in a combined fashion (Fig. 2) as done previously [3]. It appears that the effect of an addition of gentamicin to type 1 vesicles is to make the enzyme activity less susceptible to being increased by phosphatidylinositol, whereas it is the addition of phosphatidylinositol which makes the enzyme activity more susceptible to inhibition by gentamicin in type 2 vesicles.

Discussion

The results presented in this communication show that the environment of phosphatidylcholine in lipid vesicles influences markedly the activity of phospholipase A1, its regulation by the presence of negatively charged phospholipids and the nature of its inhibition by gentamicin. Considering first the data obtained in the absence of gentamicin, the present results indicate that the regulation

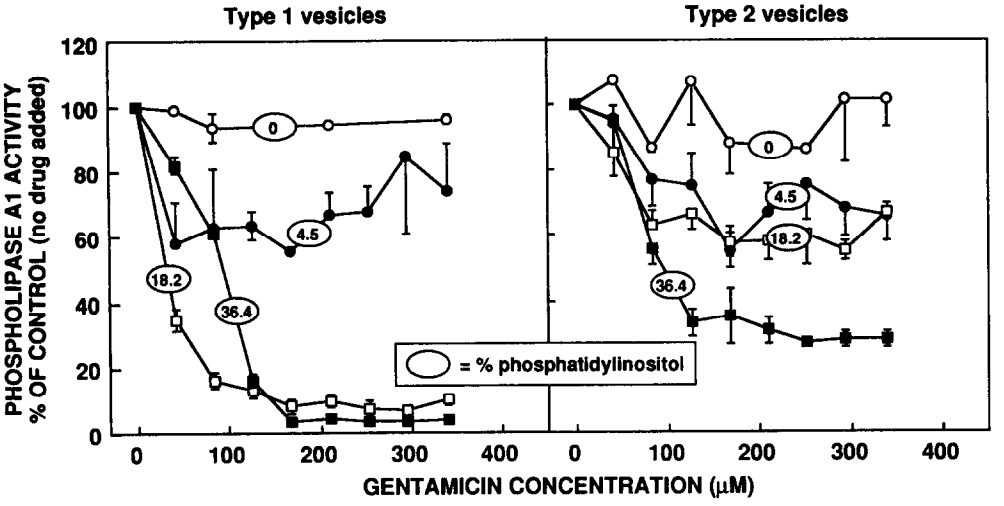

Fig. 1. Inhibition of phospholipase A1 activity by gentamicin with vesicles with increasing contents of phosphatidylinositol. Type 1 and type 2 vesicles refer to the general composition described in Table 1. The figures in the ovals on each curve indicate the phosphatidylinositol content of the corresponding vesicles (in percentage of total phospholipids; the absolute composition is given in Table 1 as A through D). Activities are expressed as percentages (±SD, N = 3) of the values observed in the absence of gentamicin (Table 2 for absolute values).

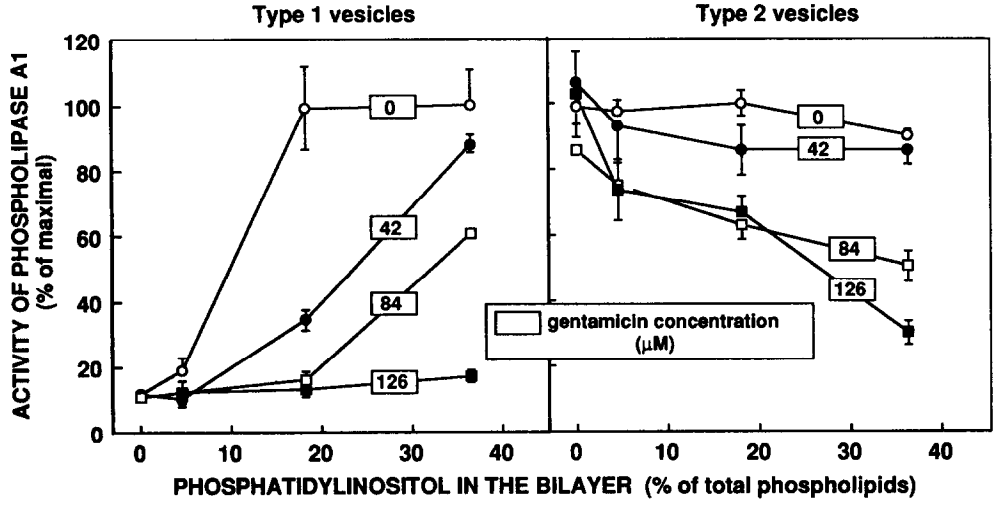

Fig. 2. Data of Fig. 1 presented to further evidence the combined influence of increases in vesicle phosphatidylinositol content (abscissa) and gentamicin concentration (figures in boxes) on phospholipase activity in relation to the models of inhibition proposed. Vesicles composition is given in Table 1 and absolute values of activities of controls (no drug added) in Table 2. See Ref. 2 for additional description and rationale of this mode of representation.

of the activity of phospholipase A1 by a negatively charged phospholipid, as evidenced by Mingeot-Leclercq *et al.* [2, 3], is only observed when cholesterol and/or sphingomyelin are also present in the vesicles, in addition to phosphatidylcholine and phosphatidylinositol. We have at present no simple explanation for this effect of the additional lipids, since their presence not only modifies the chemical composition but also the physical organization of the bilayer. Data obtained in the presence of gentamicin are compatible with the two models of inhibition proposed, namely charge neutralization [3] and substrate depletion [4], within the limits of the experimental conditions used to evidence them. Because the two sets of data could be reproduced with only one source of enzyme and a single defined substrate (1-palmitoyl-2-oleoyl phosphatidylcholine), it is clear that the differences between the two models of inhibition proposed stem from the substrate environment. Actually, the charge neutralization model could not be evidenced by Hostetler and Jellison [4] with vesicles made of phosphatidylcholine and phosphatidylinositol alone, since the enzyme does not show the necessary regulation by negative charges in this system.

Conversely, the charge neutralization model obviously takes precedence over the substrate depletion model in vesicles in which regulation of the activity of phospholipase A1 by negative charges occurs, as evidenced by the data presented here and the kinetic data reported by Mingeot-Leclercq *et al.* [3]. Further studies, however, will need to establish whether gentamicin binds in a similar fashion and to the same extent to both types of vesicles. Yet, because the composition and probably the organization of biological membranes, especially those of lysosomes, is more akin to that of type 1 than type 2 vesicles, we may already suggest that the model of charge neutralization is more relevant to the *in vivo* situation, and may therefore be of greater help in understanding and, hopefully, avoiding the phospholipidosis induced by aminoglycosides [1]. This model also suggests that the activity of phospholipase A1 may be modulated *in vivo* by variation of the lipid composition and/or the charge of the membranes, a phenomenon that may play an important role in phospholipid turnover in health and disease.

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Glucocorticoid-mediated potentiation of P450 induction in primary culture of rainbow trout hepatocytes

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Abstract—Induction of 7-ethoxyresorufin *O*-deethylase activity (a cytochrome P450IA-dependent activity) by β-naphthoflavone (0.36 μM) is increased 2–3-fold by dexamethasone or cortisol (10^{-9}–10^{-7} M) in rainbow trout hepatocyte cultures. This potentiation does not seem to be a time-dependent process and could be a classical glucocorticoid receptor-mediated event resulting in enhanced transcriptional activation of the *CYP*1A, as previously shown in mammals. Since glucocorticoid levels can increase in fish exposed to pollutants, such steroids may interfere with the induction response to xenobiotics.

The hepatic cytochrome P450-dependent monooxygenase system metabolizes a large number of potentially harmful xenobiotics, and is highly inducible by well-known organic pollutants such as polycyclic aromatic hydrocarbons (PAHs*) and polychlorinated biphenyls [1]. This system has been described well in fish species, the cytochrome P450IA (P450IA) isoenzymes being especially sensitive to these pollutants [2, 3]. In mammals, PAH-induced accumulation of P450IA has been shown to involve primarily an increase in transcription mediated by the cytosolic *Ah* receptor but posttranscriptional regulation may also be important [4, 5]. In fish, the factors involved in P450 induction by PAHs are not well characterized, e.g. the presence of an *Ah* receptor was demonstrated only recently [6]. Nevertheless, the induction response has been shown to be modulated by numerous factors such as reproduction stage, sex, age and ambient water temperature. In mammals, GC are important modulators of P450IA induction by PAH-like compounds. Both *in vivo* experiments and studies on cultured hepatocytes show a significant potentiation of the PAH-mediated induction

* Abbreviations: βNF, beta-naphthoflavone; DEX, dexamethasone; DMSO, dimethyl sulfoxide; EROD, 7-ethoxyresorufin *O*-deethylase; GC, glucocorticoids; Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid; PAH, polycyclic aromatic hydrocarbon; TAT, tyrosine aminotransferase; P450, cytochrome P450.