INTERACTION OF KANAMYCIN A AND KANAMYCIN B WITH PHOSPHOLIPIDS

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It has been suggested that the aminoglycoside drugs are ototoxic because they contain amine groups that interact with membrane phospholipids. The interaction of kanamycin A and kanamycin B with vesicles containing various phospholipids was assessed from studies of vesicle aggregation and of the fluorescence of the probes 1-anilino-8-naphthalene sulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) added to the system.

Kanamycin B, with 5 amino groups, showed a stronger interaction with the acidic phospholipids than kanamycin A, with only 4 amino groups. The evidence indicated that the interaction was an ionic one involving the charged groups of both components with penetration of the hydrocarbon interior of the bilayers. Of all the phospholipids tested polyphosphoinositide showed the greatest ability to interact with the kanamycins, supporting the proposal that interaction with this phospholipid may be the basis of the ototoxicity of aminoglycosides.

The ototoxicity of aminoglycosides is of special clinical significance because of the potential for permanent loss of inner ear function. Symptomatic ototoxicity occurs in about 2% and asymptomatic ototoxicity occurs in about 10% of treated patients[1,2]. Despite the large number of new aminoglycosides being introduced, none of them is free of ototoxic side effects.

While the structure-activity relationship of aminoglycosides has been extensively investigated, information of the structure-ototoxicity relationship is relatively less well known[3~5]. HAWKINS suggested that molecular size and configuration of aminoglycoside molecule are important for the ototoxic potential of the drug, but probably less so than the strongly basic character of the groups attached[6]. Recently, the development of several experimental models such as organ culture of the embryonic inner ear[7], perilymphatic perfusion of drugs[8] and interaction of drugs with phospholipid monolayers[9] enables the ototoxicity of different aminoglycosides to be studied in greater detail. LODHI *et al.*, from measurements of the ototoxicities of different aminoglycoside antibiotics and monitoring the cochlear microphonic potentials during cochlear perfusion in guinea pigs, concluded that the ototoxicities of different aminoglycosides correlated well with their ability to interact with monomolecular films of polyphosphoinositides[9].

Most recent work suggests that the ototoxic effects of aminoglycosides are initiated by interaction of the antibiotics with the negative-charged phospholipids in cell membrane[10,11]. Several groups have reported the binding of aminoglycosides to biological membranes, phospholipid monolayers and phospholipid bilayers. Furthermore, it has been postulated that the drug-phospholipid interaction is between the basic amino groups of the aminoglycoside molecule and the phosphates of the polyphosphoinositide molecule[11,12].

Recently we have shown that kanamycin B, with five amino groups, is more ototoxic than kanamycin A, with only four amino groups[13]. To further investigate the importance of amino groups in

the drug-phospholipid interaction, we have studied the ability of kanamycin A and kanamycin B to interact with vesicles of several phospholipids including the polyphosphoinositides. This involved a study on the kanamycin-induced aggregation of phospholipid vesicles and the usage of fluorescence probes to identify the type of interaction between kanamycins and phospholipids. We have used 1-anilino-8-naphthalene sulfonic acid (ANS) to monitor changes occuring at the surface of the vesicles[14] and 1,6-diphenyl-1,3,5-hexatriene (DPH) to assess changes occuring in the hydrophobic interior of the vesicles bilayer[15].

Materials and Methods

Materials

ANS and the phospholipids were all obtained from Sigma Chemical Co., Poole, Dorset, England. Phosphatidylserine (PS) and phosphatidylinositol-4,5-diphosphate (PIP_2) were from bovine brain and phosphatidylinositol (PI) from bovine liver, while the phosphatidylcholine (PC) was the dimyristoyl compounds. DPH was from Aldrich Chemical Co., Gillingham, Dorset, England. Kanamycin A sulfate (potency 769 μg/mg) and kanamycin B (potency 717 μg/mg) were obtained from Bristol-Myers Pharmaceuticals, Langley, Slough, England and their solutions adjusted to pH 7.4 before use.

Methods

Sonication was used to produce dispersions of mainly unilamellar phospholipid vesicles as before[16,17].

The aggregation study is based on the principle that a solution containing small phospholipid vesicles is optically clear. Addition of drugs which interact with the phospholipids induces aggregation of the vesicles and increases the turbidity of the solution.

Aggregation of the vesicles (0.5 mg/ml phospholipid except for PC/PIP_2 which were 0.25 mg/ml) was determined from the change in absorption at 400 nm 2 minutes after addition of the kanamycins[18]. The maximum dilution of the sample caused by addition of the kanamycins was about 5%. As previously noted, it was necessary to use mixtures of PS, PI and PIP_2 with PC (1 : 4, w/w) to obtain suitable rates of aggregation[16].

For the ANS fluorescence experiments, solutions of the kanamycins (10 mM) and the ANS (50 μM) were incubated together in 0.01 M Tris buffer, pH 7, for 30 minutes in the dark. The phospholipid vesicles (0.5 mg/ml phospholipid except for PIP_2 which were 0.2 mg/ml) were then added in increments to the required final concentration and the fluorescence spectrum recorded after each addition[16,19]. The maximum dilution of the sample caused by addition of the vesicles was about 2.5%. Pure phospholipids (PC, PS, PI or PIP_2) were used. ANS is useful to study the outer or hydrophilic portion of the phospholipid bilayers. ANS itself exhibits weak fluorescence in the presence of acidic phospholipids. However, the fluorescence intensity of the phospholipid-ANS system increases greatly following addition of drugs which form ionic interaction with the phospholipids.

DPH fluorescence probe is useful for the study of the inner or hydrocarbon portion of the phospholipid bilayer. DPH has the ability to insert into the hydrocarbon portion of the phospholipid bilayers and exhibits intense fluorescence. However, when polarized light is used to excite the molecule, movement of the probe inside the hydrocarbon portion results in the fluorescence becoming depolarized. Lipid bilayers with tightly packed phospholipid molecules restrict the movement of the probe resulting in less depolarization of the fluorescence. This situation may occur when a drug interacts with the hydrocarbon portion of the phospholipid bilayers[19]. The index of polarization (P) of DPH fluorescence was measured as described by WHARTON *et al.*[16]. The concentrations used (phospholipids, 0.5 mg/ml; kanamycins, 0.01 mM) were chosen so that no aggregation occurred and the samples remained optically clear. The maximum dilution of the phospholipid sample caused by addition of the kanamycins was 2%. Mixtures of PS, PI and PIP_2 (1 : 4, w/w) were used rather than the pure compounds as in the aggregation studies.

Results

Aggregation of Phospholipid Vesicles by Kanamycins

The kanamycin concentrations required to produce half the maximum aggregation of the different phospholipid vesicles are given in Table 1. No aggregation of the uncharged PC vesicles was observed at any of the kanamycin concentrations tested, whereas all the vesicles containing acidic phospholipids were readily aggregated. Vesicles containing PS show the least interaction with both drugs and the interaction increases as the negative charge on the phospholipid increases, although for kanamycin B, the difference between PI and PIP_2 is probably not significant.

Fluorescence Studies

The results obtained with the polar fluorescent probe, ANS, are given in Table 2. Examples of the curves from which the values listed in Table 2 were obtained are shown in Fig. 1. Because of quenching by the water molecules, aqueous solutions of ANS have a very low fluorescence efficiency whether kanamycins are present or not. As Table 2 shows, the fluorescence efficiency of the probe is not increased by the addition of negatively-charged phospholipid vesicles because the anionic ANS molecules cannot bind to the bilayers[19]. When kanamycin A or kanamycin B is present, addition of the phospholipids to ANS solutions results in an increase in the level of fluorescence (Fig. 1) showing that the probe can now bind. The increase in fluorescence is proportional to the amount of phospholipid present over the range of concentrations used[19] and the slopes of the lines obtained for each phospholipid are given in Table 2. Kanamycin B promotes ANS binding to all vesicles more efficiently than kanamycin A and for both drugs, the greatest effect is seen with PIP_2 and the smallest with PS as for the aggregation experiments.

Table 1. Kanamycin-induced aggregation of phospholipid vesicles[a].

Phospholipid	Concentration needed for 50% aggregation (mM)[b]	
	Kanamycin B	Kanamycin A
PC	>1	>1
PC/PS	0.036 (0.036; 0.036)	0.067 (0.065; 0.068)
PC/PI	0.023 (0.027; 0.018)	0.046 (0.044; 0.048)
PC/PIP_2	0.022 (0.025; 0.018)	0.030 (0.026; 0.034)

[a] Increasing amounts of kanamycins were added to the phospholipid vesicles (0.5 mg/ml phospholipid except PC/PIP_2 which were 0.25 mg/ml) and the extinction measured as described in Materials and Methods. Mixtures of PS, PI and PIP_2 with PC (1 : 4, w/w) were used.

[b] Defined as the concentration at which turbidity reaches half of the maximum value obtained after 2 minutes incubation with the kanamycins[18].

Values are means of determinations made on 2 separate vesicle preparations, with the individual values in parenthesis.

Table 2. Effect of phospholipids and kanamycins on ANS fluorescence[a].

Phospholipid	Fluorescence enhancement (fluorescence units per μg added phospholipid)		
	No kanamycin	Kanamycin B	Kanamycin A
PS	0	0.44	0.27
PI	0	0.77	0.50
PIP_2	0	1.625	0.85

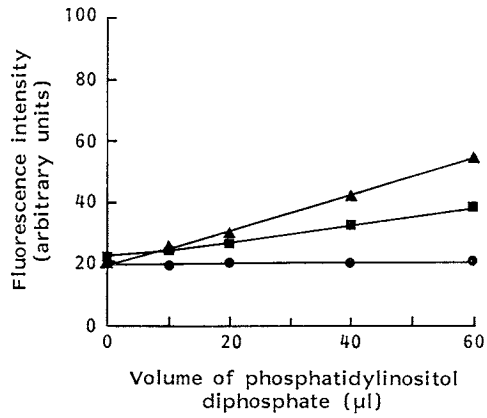
[a] Successive additions of phospholipid vesicles (0.5 mg/ml phospholipid except for PIP_2 which were 0.2 mg/ml) were made to a solution of ANS and the kanamycin as described in Materials and Methods.

The polarization of fluorescence (P), from the hydrophobic probe DPH, was used to investigate changes occurring in the hydrocarbon interior of the vesicle bilayers. The values are shown in Table 3. A higher P value represents less freedom of movement of the DPH fluorescence probe. The different vesicles give different initial values because of the differences in their molecular composition. However, the addition of aminoglycosides caused very little change in the fluorescence polarization suggesting that their effects occur mainly at the surface of the vesicles.

Fig. 1. Effect of polyphosphatidylinositol and kanamycins on ANS fluorescence.

Increasing amount of polyphosphatidylinositol vesicles were added to a mixture of ANS and a kanamycin (ANS, 50 μM; kanamycin, 10 mM) as described in Materials and Methods. The fluorescence measurements for the ANS/kanamycin B and the ANS/kanamycin A mixtures in the absence of phospholipid are 20 and 22 respectively. No kanamycin was present in the control sample.

● Control, ■ kanamycin A, ▲ kanamycin B.



Discussion

It has been suggested that amino groups play an important role in the ototoxic properties of aminoglycosides[20]. Kanamycins A and B were chosen for this study because the structure of these two aminoglycosides differ only in the replacement of a hydroxyl group in kanamycin A by an amino group in kanamycin B. We have previously reported that kanamycin B is more cochleotoxic than kanamycin A[18] and the present results show that kanamycin B interacts more strongly with acidic phospholipids than kanamycin A. LODHI *et al.* have also reported this from their studies using monolayers of phospholipids[9]. This can be explained by kanamycin B having a higher number of positive charges than kanamycin A at the physiological pH since most of the amino groups will be protonated at this pH. ALEXANDER *et al.* studied the interaction of aminoglycoside antibiotics with phospholipid liposomes using microelectrophoresis. They noticed the ability of the aminoglycosides to cause charge reversal of liposomes prepared from acidic phospholipids was ranked in the order neomycin>gentamicin>tobramycin>amikacin>kanamycin>streptomycin[21]. This order correlates with the number of strongly basic amino and guanidino groups in the aminoglycoside molecules.

Both the aggregation and the fluorescence experiments indicate that ionic bonding between the basic kanamycins and the acidic phospholipids is the basis of their interaction. There was no change in the level of DPH fluorescence (not shown) when the aminoglycosides were added which would have indicated displacement of the probe by the drugs[19] and there was no change in the degree of polarization (Table 3) to match the changes seen in the aggregation and ANS experiments.

Table 3. Effect of kanamycins on the polarization of fluorescence of diphenylhexatriene molecules in phospholipid vesicles[a].

Phospholipid	Fluorescence polarization value (P)		
	No kanamycin	Kanamycin B	Kanamycin A
PC/PS	0.29	0.254	0.26
PC/PI	0.19	0.223	0.23
PC/PIP_2	0.21	0.236	0.23

[a] 12.5 μg DPH were added to 5 mg of phospholipid in 10 ml water and the polarization of its fluorescence was measured as described in Materials and Methods before and after addition of 0.01 mM kanamycin. Mixtures of PS, PI and PIP_2 with PC (1 : 4, w/w) were used.

It may be noted that vesicle aggregation seems to depend only on the number of amine groups in the molecule. Thus kanamycin A with four amine groups is similar to the ototoxic polyamine, spermine, which also has four amine groups[17], while kanamycin B, with five amine groups, is effective at lower concentrations. On the other hand, in the ANS experiments, aminoglycosides are appreciably less efficient than simplier polyamine molecules[17]. This is probably because the bulkier ring systems of the aminoglycosides with their hydrophilic hydroxyl groups impede the insertion of the ANS ring system into the non-polar interior of the bilayer which is needed for the increase in fluorescence[14].

The phospholipid that shows the greatest interaction with the kanamycins is the most acidic, PIP_2. This agrees with the findings of other workers. ORSULAKOVA *et al.* studied ^{32}P-labelling of different phospholipids of guinea pig inner ear tissues after treatment with neomycin for three weeks and they reported that the labelling of PIP_2, but not other phospholipids, was affected by the aminoglycoside[22]. WANG *et al.* studied the effect of neomycin on monomolecular films of different phospholipids and they observed an interaction only in the PC/PIP_2 films[23]. Hence our results support the current theory that acidic phospholipids, especially PIP_2, are crucially involved in the toxicity of aminoglycosides in the inner ear. Almost all of these phospholipids are present on the inner surface of the plasma membrane of mammalian cells[24]. The negative charges on the outside of the cell are from the carboxylate groups of sialic acids which interact with aminoglycosides less strongly than phosphate groups[25]. Aminoglycosides will decrease the cochlear microphonic potentials within minutes of introduction into the perilymphatic spaces of the guinea pig cochleas[26]. Thus if the damaging interaction is with the phospholipids on the inner surface of the membrane, the drugs must cross the bilayer rapidly.

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