INTERACTION OF AMINOGLYCOSIDE ANTIBIOTICS WITH PHOSPHOLIPID LIPOSOMES STUDIED BY MICROELECTROPHORESIS

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(Received for publication March 1, 1979)

Microelectrophoresis was found to be a rapid method for studying the interaction between aminoglycoside antibiotics and liposomes prepared from acid phospholipids. The ability to cause charge reversal of liposomes prepared from phosphatidyl inositol was ranked in the order neomycin > gentamicin > tobramycin > amikacin > kanamycin > ribostamycin > streptomycin > dihydrostreptomycin > Ca^{2+}. Similar results were obtained with liposomes prepared from a mixture (8:2) of phosphatidyl choline and phosphatidic acid, but no effect was detectable with neutral liposomes made from phosphatidyl choline only. The results support the view that the attraction between positively charged nitrogen groups on the antibiotics and the negatively charged groups of acidic phospholipids are predominantly responsible for the interaction. Extension of the studies to ionic strengths and calcium concentrations similar to those found *in vivo* showed a reduction, but not elimination, of the observed effects.

KUNIN[1] estimated the tissue binding of several antibiotics by measuring the inhibition of their antibacterial activity in the presence of various tissue homogenates and cell components. He found that the antibacterial activity of aminoglycoside antibiotics (5 μg/ml) was not inhibited by the presence of phosphatidyl inositol (1 mg/ml). AUSLANDER *et al.*[2] did not detect any influence of gentamicin on the surface pressure of monomolecular films of zwitterionic (neutral) and positively charged phospholipids, or stearic acid at pH 5. However, they found a small increase in the pressure of stearic acid films in the presence of gentamicin at pH 7 and 8. Formation of a complex between positively charged gentamicin and negatively charged stearate ions would be consistent with the observed pH-dependent effect. Further, CORRADO *et al.*[3] reported that aminoglycosides inhibited the phospholipid-facilitated diffusion of cations across a model membrane, presumably by binding to the acidic groups of the phospholipids with a higher affinity than the inorganic cations. They also showed that, among the phospholipids present in the model membrane, phosphatidyl inositol was particularly active in binding calcium ions. ORSULAKOVA *et al.*[4] reported competitive binding of neomycin and calcium to inner ear tissue homogenates, and suggested that a complex is formed between the antibiotic and polyphosphoinositides (and other polyanions) contained in the tissue. The same group[5] found that addition of neomycin increased the turbidity of a mixture of polyphosphoinositides at low ionic strength. We found (A. PENDREICH and I. GONDA, unpublished results) similar turbidity changes with phosphatidic acid but the measurements were rather sensitive to the degree of mechanical agitation of the suspension. This would be typical for charged colloidal systems whose stability depends on the concentration of counterions. Neutralisation of charge, *i.e.* reduction of the stabilising repulsions of the electric double layer[6] leads to onset of particle aggregation. Micro-electrophoresis—an electrokinetic technique[6,7]—can be used to investigate the changes in the electric

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double layer of multilamellar phospholipid vesicles (liposomes)[7]. In view of the large size of these vesicles compared to the thickness of the double layer, the electrophoretic mobility obtained from microelectrophoresis is independent of the particle size.[6] Hence, the polydispersity of liposomes would not affect the findings reported below.

Materials and Methods

The antibiotics were obtained from Mead Johnson Laboratories Ltd. (amikacin base), Sigma Chemical Co. (sulphates of dihydrostreptomycin, kanamycin, neomycin and streptomycin), Nicholas Laboratories Ltd. (gentamicin sulphate), Morrith Laboratories Ltd. (ribostamycin sulphate) and Lilly Research Centre Ltd. (tobramycin base). Pure synthetic D,L-3-lecithin (phosphatidyl choline) (PC) and the sodium salt of phosphatidic acid (PA) were purchased from Koch-Light Laboratories, and phosphatidyl inositol (PI) from Lipid Products. Although no further purification was attempted, all chemicals used were of the highest grades commercially available.

Tris buffer, pH 7.4, ionic strength I=0.092, was prepared from Tris (hydroxymethyl) methylamine and 0.1 N hydrochloric acid. Tris buffers with different ionic strengths were prepared by 1: 1 dilution of the original buffer with double distilled water and addition of an appropriate amount of sodium chloride.

Multilamellar liposomes[7] were obtained as follows: A chloroform solution of the phospholipid (2.5 mg/ml) was prepared, flushed with nitrogen and stored at −16°C. The required amount of this solution(-s) was placed in a round-bottom flask and the solvent removed under reduced pressure in a rotary evaporator at 37°C. An amount of tris buffer to give a suspension of lipid 1 mg/ml was added to the thin film formed in the flask, the mixture was then hand-shaken until dispersed. Mechanical agitation, using a Whirlimixer (Fisons, Loughborough, U.K.) was applied to the flask for one minute. The liposome preparations were kept when necessary for up to 3 days at 4°C.

Stock solutions of antibiotics were prepared in the tris buffer. The molarities were calculated from the base contents of the supplied antibiotics, taking the following molecular weights for the free bases: amikacin 585.8, dihydrostreptomycin 583.6, gentamicin 500, kanamycin 484.5, neomycin 774, ribostamycin 454.5, streptomycin 581.6 and tobramycin 467.5.

Aliquots of stock solutions of the aminoglycosides and/or calcium chloride were added to 1 ml of the liposome suspension and the mixture was made up to 10 ml with a buffer to give the required concentration of aminoglycosides, calcium and phospholipid (0.1 mg/ml).

Electrophoretic measurements were made at 25°C using a Rank microelectrophoresis apparatus, mark II (Rank Brothers, Bottisham, Cambridge, U.K.) with a cylindrical cell and platinum black electrodes.[8] The system was illuminated with a quartz iodine lamp, and the scattered light observed perpendicularly to the incident beam by a microscope focused at the stationary level.[6] Ten liposomes were timed over a fixed distance on the calibrated eyepiece; polarisation of electrodes and errors due to drifting were minimised by timing each liposome in both directions of the electric current. The electrophoretic mobility (U) is related to the experimental parameters by

$$U=\frac{V}{E}$$

where V, the velocity, is the graticule distance divided by the time of passage, and E is the field strength calculated from the applied voltage divided by the distance between electrodes. The voltage was varied to give timings of about 10 seconds.

Results

All aminoglycoside antibiotics tested reduced the electrophoretic mobility of PI liposomes in tris buffer, I=0.092 (Fig. 1); the mobility of these liposomes in the absence of antibiotics was $(-3.35\pm 0.10)\times 10^{-8}$ m^2 s^{-1} V^{-1}. On an equimolar basis, the reduction in mobility was quite substantial com-

Fig. 1. The effect of aminoglycoside and calcium concentrations (C) on the electrophoretic mobility (U) of phosphatidyl inositol liposomes in tris buffer (pH 7.4, I=0.092 M, 25°C).

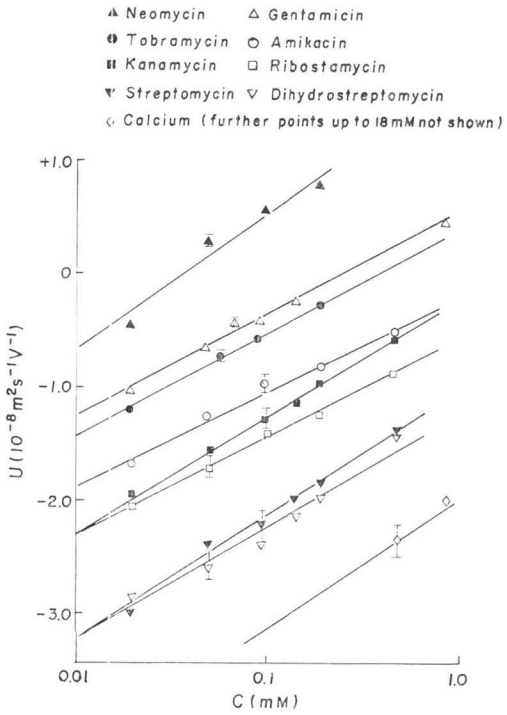


Table 1. Charge reversal concentrations of aminoglycoside and calcium ions in tris buffer at 25°C.

Cation	Phospholipid	Ionic strength	Charge reversal concentration (mM)
Neomycin	PI	0.092	0.04
Gentamicin			0.22
Tobramycin			0.38
Amikacin			1.80
Kanamycin			1.89
Ribostamycin			4.55
Streptomycin			9.19
Dihydrostreptomycin			16.26
Calcium			73.62
Neomycin	PC: PA (8: 2)	0.092	1.92
Gentamicin			2.64
Kanamycin			20.35
Streptomycin			41.90
Dihydrostreptomycin			119.90
Gentamicin	PI	0.146	0.69
Calcium			208.00
Gentamicin (+Calcium 2.5 mM)			1.05

pared to the effect of calcium ions. High concentrations of neomycin and gentamicin actually caused reversal of the charge on liposomes as indicated by the positive electrophoretic mobility. However, frequently the velocities were too low to measure at high antibiotic concentrations and charge reversal would have required excessive quantities of the weakly interacting substances. The charge reversal concentrations, which are the concentrations required to produce zero mobility[9] were calculated using linear regression analysis. These results together with data obtained from other experiments reported below are presented in Table 1.

The findings in experiments with liposomes prepared from a mixture (8: 2) of a neutral phospholipid PC and the negatively charged acid PA, under the same conditions as described above, show a similar trend (Fig. 2); these liposomes had an electrophoretic mobility of $(-1.26 \pm 0.20) \times 10^{-8}$ m^2 s^{-1} V^{-1} at I=0.092 in the absence of antibiotics. The charge reversal concentrations calculated from the least mean square line are also presented in Table 1.

Liposomes prepared from pure PC had zero mobility in the tris buffer, pH 7.4, I=0.092, 25°C; this is in agreement with other authors[10]. The addition of neomycin, the antibiotic which had the largest effect in other experiments, caused no detectable change in mobility of the PC liposomes. It is therefore unlikely that neomycin was adsorbed at the surface of these liposomes.

An increase in ionic strength by addition of an 'inert' salt would be expected to modify the results by two mechanisms: one is to weaken the attraction between the oppositely charged drug and phospholipid ions, and the second one is to compress the double layer surrounding the liposomes.

Fig. 2. The effect of aminoglycoside concentration (C) on the electrophoretic mobility (U) of mixed phosphatidyl choline/phosphatidic acid (8: 2) liposomes in tris buffer (pH 7.4, I=0.092 M, 25°C).

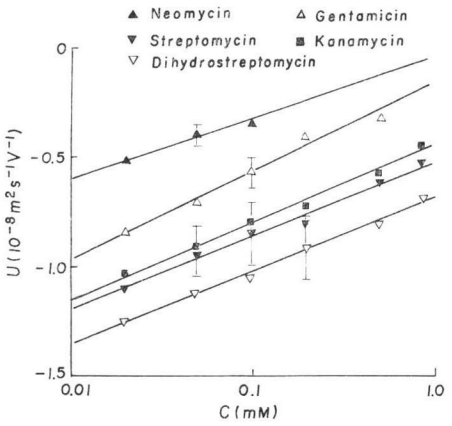


Fig. 3. The influence of ionic strength and calcium ions on the electrophoretic mobility (U) of phosphatidyl inositol liposomes at various concentrations (C) of gentamicin; tris buffer (pH 7.4, 25°C).

Symbols for ionic strength are

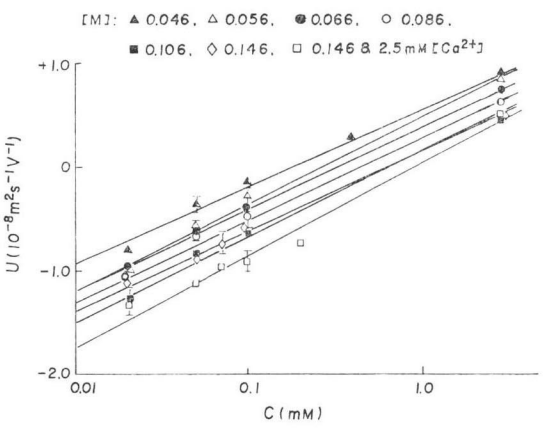
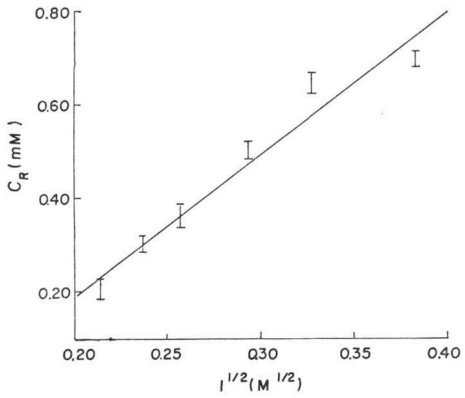


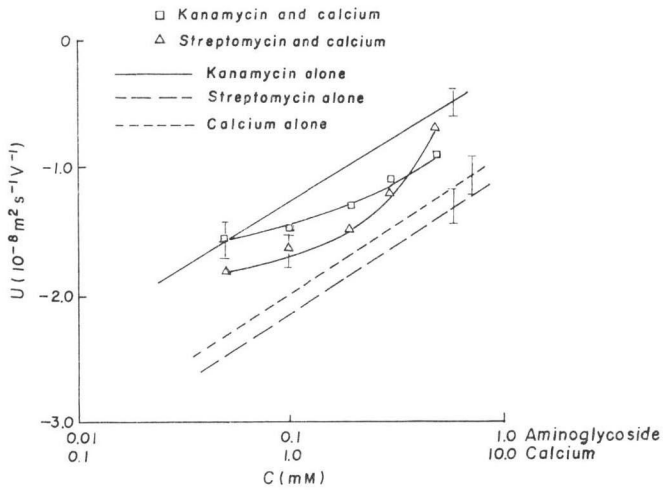
Fig. 4. The dependence of charge reversal concentration (C_R) of gentamicin on the square root of ionic strength ($I^{1/2}$); phosphatidyl inositol liposomes in tris buffer (pH 7.4, 25°C, I=0.046 M) adjusted with sodium chloride to required ionic strength.



Since both mechanisms would lead to a reduction of the observed effects of aminoglycosides, we studied the ionic strength dependence of the electrophoretic mobility of PI liposomes in the presence of various concentrations of gentamicin. It is seen (Fig. 3) that even at the highest ionic strength (c.f. isotonic saline), gentamicin had a marked influence on the mobility at concentrations found *e.g.* in the kidneys of patients receiving gentamicin therapy[11] and well within the range of concentrations found in animal experiments[12,13]. The charge reversal concentration of gentamicin is approximately a linear function of the square root of the ionic strength of the buffer (Fig. 4). If we included the contribution of the antibiotic to the ionic strength, the line would be slightly shifted towards the right side of the diagram.

Further, the effect of calcium ions on the mobility of liposomes was investigated. The line shown in Fig. 1 was calculated from results obtained at six concentrations of calcium chloride in the range 0.5 ~ 18.0 mM. The presence of 2.5 mM Ca^{2+} (*e.g.* found in human blood serum[14]) weakened the effect of gentamicin on the mobility of PI liposomes (Fig. 3 and Table 1). In order to assess whether calcium could actually eliminate the observed effects of the 'weaker' antibiotics in particular, mixtures of calcium chloride with kanamycin or streptomycin were added to PI liposomes. It can be seen (Fig. 5) that calcium behaved as a weak competitor for the interaction with liposomes (note the two concentration scales). In the region of 1 ~ 10 mM Ca^{2+}, the effect of kanamycin on mobility was slightly reduced whereas the combination of calcium with streptomycin produced a greater reduction

Fig. 5. The effect of calcium and aminoglycosides on the electrophoretic mobility (U) of phosphatidyl inositol liposomes in tris buffer (pH 7.4, I=0.092 M, 25°C).



of mobility than either of the ions alone.

Discussion

The ability of aminoglycoside antibiotics to reduce the electrophoretic mobility of liposomes containing acid phospholipids was found to be in the order [neomycin] > [gentamicin] > tobramycin > amikacin > [kanamycin] > ribostamycin > [streptomycin] > [dihydrostreptomycin]: the brackets indicate that the drugs were tested with both types of negatively charged liposomes (Figs. 1 & 2, Table 1). The effect was reduced, but not eliminated, at ionic strength approximately equal to that of isotonic saline (Figs. 3 & 4). Calcium at concentrations found commonly *in vivo* reduced the influence of kanamycin and slightly increased the effect of streptomycin. This observation is consistent with a competition between calcium and the antibiotics for the phospholipids. All tested antibiotics exhibited a much stronger reduction of electrophoretic mobility than calcium ions on an equimolar basis. It is interesting that the local anaesthetics dibucaine, tetracaine, cocaine, lidocaine and procaine exerted a smaller effect than calcium ions on the mobility of negatively charged liposomes made from phosphatidyl serine[15], under conditions otherwise similar to those in our experiments.

DEGUCHI *et al.*[16] found a similar order to us in binding of aminoglycosides to acidic mucopolysaccharides. These workers observed, however, that the interactions were practically eliminated at physiological pH and ionic strength. KUNIN[1] pointed out that the strength of binding of aminoglycosides to tissue homogenates correlates well with the number of strongly basic amino- and guanidino- groups they contain: Neomycin has 6, kanamycin B, gentamicins C and tobramycin have 5, kanamycins A and C, amikacin and ribostamycin have 4, streptomycin and dihydrostreptomycin have 3 basic amino groups.[17] It is therefore tempting to suggest that our observations and those of DEGUCHI *et al.*[16] can be explained largely in terms of interaction between the positively charged nitrogen groups on the antibiotics and the negatively charged groups on the acidic phospholipids and mucopolysaccharides. This suggestion is supported further by the following:

1. examination of the data of AUSLANDER *et al.*[2] indicates that gentamicin interacts with stearic acid only at pH where both species are significantly ionised.
2. neomycin does not appear to interact with neutral PC liposomes.
3. increasing the ionic strength reduces the interaction[16] (Figs. 3 & 4 and Table 1).

It was possible to increase the concentrations of neomycin and gentamicin to levels where charge reversal was observed (Figs. 1 & 3). This phenomenon is usually taken to be an indication of

specific adsorption of the counterions to the surface of a charged particle; it is observed commonly with large organic counterions which can interact by polarisation and VAN DER WAALS forces in addition to charge interactions.[18] However, in view of the high positive charges on neomycin and gentamicin, electrostatic forces are likely to dominate. Yet, we note in this context that the charge reversal concentration of gentamicin in tris buffer of 'full strength' (I=0.092, see Fig. 1 and Table 1) was substantially lower than what we would expect from Figs. 3 or 4 ('half-strength' tris buffer adjusted to required ionic strength with NaCl). Although we have not investigated this phenomenon any further, it would appear that the large cation of tris (hydroxymethyl) methylamine has a much more pronounced effect on electrophoretic mobility than the sodium ion used to adjust the ionic strength in the latter experiments.

The neutralisation of surface charge of PI and (PC+PA) liposomes by aminoglycosides is consistent with the observed physical metastability of the system alluded to in the introduction. A neutral complex formed between acidic phospholipids and cations, either calcium or aminoglycosides, is much more lipophilic than its constituents (I. GONDA, unpublished observations). The cation-carrier properties of acidic phospholipids[3] could be therefore explained in terms of the lipophilicity of the complex. Furthermore, the observed lack of inhibition of antibacterial activity of aminoglycosides by phosphatidyl inositol[1] could be, perhaps, due to the fact that the drug-phospholipid complex is actually transported more readily into the bacteria than the free drug.

Charge neutralisation, however, occurs at high antibiotic concentrations, whereas competition with calcium and reduced mobility are observed at much lower concentrations. Local anaesthetics also compete with calcium for binding and reduce the electrophoretic mobility in acid phospholipid liposome systems[15,19]. These effects have been correlated with changes in membrane permeability to cations[15]. It remains to be seen whether such an action could help to explain the variety of toxic effects of aminoglycoside antibiotics[20].

Acknowledgement

The authors wish to thank Dr. B. A. HEMSWORTH and Mr. D. A. RAWLINS for help and advice, and the Lilly Research Centre Ltd., Mead Johnson Laboratories Ltd. and Nicholas Laboratories Ltd. for the supply of the antibiotics.

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