Freeze-fracture has been used to examine the effects of gentamicin on membrane structure in liposomes of different anionic phospholipids combined with a neutral phospholipid, phosphatidylcholine. The molar ratios of neutral: anionic lipid were 1:1 (high anionic lipid ratio) and 4:1 (low anionic lipid) and the liposomes were incubated with 0.1 mM (low) and 1 mM (high) gentamicin. With the anionic phospholipid phosphatidylinositol bisphosphate, an identifiable disruption of the membrane bilayer was observed as well as aggregation of liposomes leading to membrane fusion. These effects occurred both at low gentamicin concentration and low anionic lipid content of the liposomes; these responses were not inhibited by 1 mM Ca\(^{2+}\). With the other anionic lipids tested (phosphatidylserine, phosphatidylinositol and phosphatidylinositol monophosphate), only aggregation and fusion of liposomes was observed and this effect only occurred at high gentamicin concentration and high anionic lipid content. Further, 1 mM Ca\(^{2+}\) inhibited the responses of these other anionic lipids to gentamicin. The results demonstrate the unique character of the interaction between gentamicin and phosphatidylinositol bisphosphate and provide further support for the hypothesis that a specific binding to this lipid is a key step in the ototoxic action of aminoglycoside antibiotics. They also suggest that such an interaction in vivo might cause alterations to the structure and properties of cell membranes in the inner ear.

**Gentamicin alters membrane structure as shown by freeze-fracture of liposomes**

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**Introduction**

A significant factor in the toxic action of aminoglycoside antibiotics is thought to be an interaction of these drugs with anionic sites at the cell plasma membrane, and in particular with the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (Schacht, 1986). Studies in vivo have shown that aminoglycosides inhibit metabolism of this lipid in both inner ear and kidney tissues (Schacht, 1976; Tou et al., 1972), the two principal sites which are susceptible to the toxic side effects of the drugs. In vitro experiments have also been conducted using liposomes, a model membrane system composed of vesicles formed from lipid bilayers of defined composition. These studies have demonstrated that aminoglycosides cause unique alterations to the physicochemical properties of membranes that contain PIP\(_2\). Amongst the changes noted are lowering of the phase transition temperature ('melting temperature') of the lipids (Wang et al., 1984), clustering of lipids within the membrane (Au et al., 1986) and leakage of contents from the aqueous space enclosed within the liposome (Au et al., 1987). These alterations were either not seen or were seen to a much smaller extent with other anionic phospholipids and the magnitude of the effects produced on PIP\(_2\)-containing liposomes by a series of different aminoglycosides correlated with the relative ototoxicity of each drug. This suggests a relation between the drug-PIP\(_2\) interaction and the adverse effects produced in the ear.

The physicochemical effects produced suggest that the drug-PIP\(_2\) interaction alters membrane...
structure. The structure and structural changes of both biological and artificial membranes can be analysed using freeze-fracture as characteristic patterns of fracture are determined by the physical arrangement of the lipids in the membrane (Deamer et al., 1970; Pinto da Silva, 1971; Forge et al., 1977). It is also possible to identify reactions between drugs and specific lipids. This can be used to determine the localisation of the lipid(s) (Severs and Robenek, 1983). For example, the anti-mycotic agent filipin binds to cholesterol to form visible, discrete deformations in the plane of the membrane (Severs and Robenek, 1983; Forge, 1985) and the polypeptide antibiotic polymyxin interacts with anionic phospholipids to form readily recognisable membrane perturbations (Bearer and Friend, 1982).

The present work was initiated to determine by freeze-fracture of liposomes the effect on membrane structure produced by the binding of gentamicin to PIP₂. Such knowledge might provide a basis for identifying the site(s) of interaction within inner ear tissue and test the hypothesis (Schacht, 1986) of a drug-PIP₂ interaction in ototoxicity. However, aminoglycosides are expected to interact electrostatically with all anionic lipids; so, in order to differentiate any specific action with PIP₂, the effects of gentamicin on a number of different anionic lipids were compared. In addition, as non-specific interactions between gentamicin and anionic lipids should be antagonised by calcium, the effects of this cation on the drug-lipid interactions were also tested.

**Materials and Methods**

1-α-phosphatidylcholine (PC), Distearylphosphatidylcholine (DSPC), 1-α-phosphatidylserine (PS), and 1-α-phosphatidylinositol (PI) were purchased from Sigma Chemical Co. Phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP₂) were either isolated from Sigma brain extract by chromatography on immobilised neomycin (Schacht, 1978) or purchased directly from Sigma. The purity of the lipid was determined by thin layer chromatography before use. Gentamicin sulphate (Gm) was supplied by Kirby-Warrick Ltd and the Schering Corp. The buffer used throughout the experiments was 0.05 M HEPES (N-2-hydroxyethyl-1-piperazine-N'2-ethanesulphonic acid), pH 7.0.

**Preparation of liposomes**

Liposomes were formed from a mixture of one of the anionic phospholipids (PS, PI, PIP or PIP₂) with a neutral phospholipid (either PC or DSPC). The molar ratio of neutral : anionic lipid was either 1 : 1 or 4 : 1. PC-only and DSPC-only liposomes were prepared as controls. The neutral phospholipids do not bind to aminoglycosides and act to dilute charges and stabilise the liposomes; in natural membranes, neutral phospholipids constitute 40–70% of the phospholipids.

The lipid mixture dissolved in chloroform was placed in a round bottom flask and the chloroform evaporated by rotation under nitrogen to produce a uniform film of dry lipid. Residual solvent was removed by storing the flask overnight under vacuum. The dried lipids were then suspended in HEPES buffer and vortexed intermittently for one hour.

**Gentamicin and calcium treatments**

Each liposome preparation, at a final concentration of approx. 15 μmol lipid ml⁻¹ was incubated with either i) 0.1 mM Gm ii) 1 mM Gm iii) 1 mM Ca²⁺ iv) 0.1 mM Gm + 1 mM Ca²⁺ or v) 1 mM Gm + 1 mM Ca²⁺ for 1 h. Liposomes containing PC were incubated at room temperature, those containing DSPC at 60°C (Wang et al., 1984) because of the higher transition temperature of the latter.

**Freeze-fracture**

Small drops of each incubation mixture (approx. 1–2 μl) were placed on ridged, gold specimen supports or were sandwiched between two copper plates for fracture in a double replica device. Samples were frozen in solidifying Freon 22 or by rapid plunging into a constantly stirred mixture of propane:isopentane (3 : 1) cooled by liquid nitrogen using an adaptation of the slam freezing device designed by Boyne (Phillips and Boyne, 1984). The appearance of the liposome preparations was the same following either of these procedures and showed no evidence of any structural distortions due to the formation of large ice-crystals during freezing. It was also found that
PS-PC liposome preparations frozen by either of the above methods appeared no different from samples prepared with glycerol (25% v/v) added as cryoprotectant before freezing, or from those frozen by plunging into liquid nitrogen slush or by slamming against a polished copper block cooled by liquid nitrogen.

Fracture was performed on either a Balzers BAF 301 apparatus or a Balzers BAF 400D machine at a temperature of \(-115^\circ C\). For every sample at least two replicas were prepared, one of which was of a specimen that had been etched for 1 min before replication. On each freeze-fracture run, with the samples on single specimen supports, three different samples were prepared simultaneously, and for those in double replica holders, five different samples were prepared at the same time. This enabled control of possible non-optimal conditions in the freeze-fracture device affecting the appearance of the fracture faces (for example the deposition of condensation on the exposed surfaces). Replicas were floated free on distilled water and cleaned in 40% chromic acid.

Results

Control preparations: PC and DSPC liposomes and untreated mixed lipid liposomes

All the untreated liposome preparations (i.e. those incubated with neither calcium nor gentamicin) had a generally similar overall appearance. They consisted of vesicles of a wide range of sizes, randomly dispersed throughout the sample (Fig. 1). The smallest liposomes appeared to be single bilayer vesicles whilst the larger bodies were composed of multibilayers. There were

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Fig. 1. PS:PC (1:1) Liposome preparation. Liposomes are of various sizes. Smaller ones (arrowed) appear as unilamellar vesicles and larger ones show corrugated fracture faces. Mag. ×11300; Bar = 0.5 μm.
however, differences in the detailed appearance depending on their composition. PC-only and DSPC-only multibilayer liposomes are shown in Figs. 2 and 3, respectively, where the multibilayer form is characterised by the presence of concentric fracture steps. In PC (natural egg lecithin) the hydrocarbon chains of the phospholipid molecules are in a fluid (liquid crystalline) phase and this is reflected in the random pattern of the fracture from one bilayer to the next (Fig. 2). Alternatively, in the synthetic analogue DSPC, the hydrocarbon chains of the phospholipid are all derived from the same fatty acid, stearic acid, which is in a solid 'gel' phase at the experimental temperatures. In freeze-fracture replicas, this is evident by the regular appearance of the fracture steps and the fact that fracture from one bilayer to the next occurs relatively infrequently (i.e. there are fewer steps exposed) (Fig. 3). This is because the fracture plane within a single bilayer is more readily perpetuated in the gel phase where there is ordered packing of the lipid molecules, than in the liquid-crystalline phase where the packing of lipid molecules is random.

The addition of an anionic phospholipid caused changes in the appearance of the liposome fracture faces. For example, in PC-PS (molar ratio 1:1) preparations (Fig. 1), the multibilayer liposomes showed irregular convolutions. In most of the liposomes composed of DSPC : anionic phospholipid at either 1:1 or 4:1 molar ratios, the fracture faces had a regular pattern of alternate thick and thin lines (Fig. 4). Only 1:1 mixtures of DSPC : PIP2 were different (Fig. 5); these liposomes often assumed an irregular shape, were smooth-surfaced and showed very few fracture planes. These features suggest an unusually solid gel phase in PIP2 : DSPC bilayers. This result is in agreement with data derived from differential scanning calorimetry which showed that PIP2 was the only anionic lipid to raise the phase transition temperature of DSPC (Wang et al., 1984).

Effects of gentamicin and calcium

Controls: neutral lipids. The appearance of neither the PC-only nor the DSPC-only preparations were affected by incubation in the presence of gentamicin, Ca2+ or gentamicin + Ca2+. The liposomes showed no structural alterations and remained randomly dispersed.

Neutral + anionic phospholipid (4:1 molar ratio). Gentamicin had no observable effect on either PS- or PI-containing liposomes when the molar fraction of these phospholipids in the bilayers was 1:4. PIP-containing liposomes showed a tendency to clump together in the presence of 1 mM gentamicin (Fig. 6) but this response did not occur with 0.1 mM gentamicin; it was also unaffected by the addition of calcium. PIP2-containing liposomes in the presence of 1 mM gentamicin showed a marked aggregation and frequent irregular fractures occurred within the bilayers (Fig. 7). These features were already present when the liposomes were exposed to 0.1 mM gentamicin. Even with the additional presence of 1 mM calcium at this lower gentamicin concentration, discontinuous fractures within the lipid aggregates were clearly observable (Fig. 8).

Neutral + anionic lipids (1:1 molar ratio). With high concentrations of anionic lipid in the bilayers, 1 mM gentamicin produced an extensive

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**Fig. 2.** PC multibilayer liposome. The liposome is composed of concentric bilayers which appear after fracture as a series of concentric steps (arrows). The fracture faces are smooth and fracture occurs frequently and randomly from one bilayer to the next. Mag. ×18000; Bar = 0.5 μm.

**Fig. 3.** DSPC liposome. Fracture occurs less frequently than with PC and the fracture steps (arrows) are regular. Mag. ×27000; Bar = 0.25 μm.

**Fig. 4.** PS : DSPC (1:4). Fracture faces show regular alternate thick and thin lines. Mag. ×54000; Bar = 0.1 μm.

**Fig. 5.** PIP2 : DSPC (1:1). Liposome is irregular in shape and fracture from one bilayer to the next occurs only rarely. Mag. ×18000; Bar = 0.5 μm.
aggregation of the liposomes with all the anionic lipids tested. In the PS- and PI-containing liposomes, which responded similarly in all incubations, the liposome aggregates had the appearance of two-dimensionally extensive multibilayer sheets (Fig. 9) suggesting that liposomes had fused. Aggregation was much less pronounced with 0.1 mM gentamicin (Fig. 10). The effects with PIP were essentially similar, except that the aggregates showed large numbers of small, closely packed vesicle-like structures (Fig. 11). Whether these represented incomplete fusion of small liposomes or the breakdown of large bodies into smaller ones could not be determined. With PIP₂, however, the gentamicin had an additional, specific effect which suggested that reorganisation and disruption of bilayers had occurred. Within the lipid aggregates there were numerous distinct discontinuous fractures in liposomes prepared with PC (Fig. 12) and in liposomes prepared with DSPC, bilayer sheets appeared to have been replaced by small, closely packed micellar-like structures (Fig. 13).

Calcium alone produced no obvious effect in any liposome preparation. The presence of calcium, however, inhibited the response of PS and PI to gentamicin: the liposomes were aggregated but they maintained their individuality, and the extensive multibilayer sheet-like forms were not evident (Fig. 14). With PIP there was little noticeable effect of calcium on the response to 1 mM gentamicin. With PIP₂ liposomes, both the gentamicin-induced aggregation and bilayer disruption remained extensive in the presence of calcium (Fig. 15). Indeed, calcium appeared to enhance the effect of the gentamicin on PIP₂ since even at 0.1 mM gentamicin with 1 mM calcium both aggregation and the formation of micelle-like structures were marked (Fig. 16).

Discussion

The present work illustrates that gentamicin uniquely alters the structure in lipid bilayers containing PIP₂ and that freeze-fracture is an adequate means to characterise these effects. In addition, gentamicin can induce identifiable alterations to membranes containing anionic lipids in general. However, this non-lipid-specific effect, a close association of membranes which may lead to membrane fusion, is obtained only at high gentamicin concentrations and high anionic lipid ratio, and it is inhibited by calcium. These characteristics imply that the non-specific response may be the consequence of electrostatic interactions between the cationic drug and anionic phospholipid. In the toxic action of the aminoglycoside this could represent an acute and reversible phase as suggested earlier (Schacht, 1986). With PIP₂ there is not only an aggregation but also an obvious disorganisation of the bilayer which is most readily seen as the formation of micelle-like structures within the liposome aggregates. These phenomena can even be observed at low drug or low phospholipid concentration and calcium does not appear to inhibit the responses. This demonstrates the uniqueness of the interaction between the aminoglycoside and PIP₂ vis a vis other anionic phospholipids.

The unique character of the gentamicin-PIP₂ interaction has previously been deduced from specific physicochemical effects of aminoglycosides on this particular phospholipid. The present study is entirely consistent with those observations and suggests a structural basis for earlier interpretations of changes in lipid phase and increases in membrane permeability (Wang et al., 1984; Au et al., 1986, 1987). The small structures which appear

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Fig. 6. PIP : DSPC (1:4) with 1 mM gentamicin. Some aggregation appears to be present but the individual liposomes are identifiable and there is no indication of fusion. Mag. x 22700; Bar = 0.25 μm.

Fig. 7. PIP₂ : DSPC (1:4) with 1 mM gentamicin. There is quite marked aggregation and most liposomes are very closely associated suggesting fusion. Within the aggregates, there are many small structures. Mag. x 45000; Bar = 0.2 μm.

Fig. 8. PIP₂ : DSPC (1:4) with 0.1 mM gentamicin and 1 mM Ca²⁺. Aggregation and fusion of liposomes are apparent and there are numerous small fracture steps indicating a change from the normal bilayer form. Mag. x 50000; Bar = 0.2 μm.
in the lipid aggregates resemble micelles and the series of parallel lines within lipid aggregates (e.g. in Fig. 12) are reminiscent of the freeze-fracture appearance of a hexagonal phase structure. Micellar and hexagonal phases are both structural alternatives to the lamellar (bilayer) phase which may be adopted by lipids in an aqueous environment (Reiss-Husson and Luzzati, 1966; Verkleij, 1984). Such changes in the organisation of the lipid molecules within a membrane are likely to have significant effects on the properties of the membrane and may explain the drug-induced changes in membrane permeability.

While alterations of bilayer structure could be visualised in these liposomes, the localisation of the drug-lipid interaction in cochlear hair cells poses further problems. Firstly, unlike the membrane perturbations caused by filipin or polymyxin in natural membranes, the gentamicin-PIP\textsubscript{2} interaction does not produce a discrete lesion (as filipin) nor a characteristic patterning on fracture faces (as polymyxin). Further, the considerable differences between liposomes and biological membranes must be emphasised. The bilayers in the liposomes are composed of only two lipid species which are presumed to be distributed evenly along each leaflet of the bilayer and symmetrically between the inner and outer leaflet. The lipid composition of the membranes of hair cells is not known, but biological membranes in general are composed of a variety of phospho- and other lipids many of which may be localised to particular domains in the membrane and be asymmetrically distributed between the two membrane faces. Indeed it is thought that PIP\textsubscript{2} is normally present only in the inner leaflet of the plasma membrane in most cells and represents a relatively small proportion of the total cell membrane phospholipid (Berridge, 1986; Berridge and Irvine, 1984). These factors may influence the sensitivity of freeze-fracture to localise gentamicin-induced lesions in cells of the inner ear.

Conversely, it should be noted that various changes to the membranes of cells in the cochlea have been observed following chronic gentamicin treatment. Interestingly, in the light of the apparent fusion of liposomes seen in this study, stereocilial fusion has often been reported to be an early effect of aminoglycoside treatment. Recent freeze-fracture studies of the organ of Corti of the guinea pig (McDowell and Forge, in preparation) have shown that gentamicin treatment changes the distribution of intramembrane particles (proteins) in the stereocilia indicating disturbances of membrane structure. Drug-induced alterations of the lateral membrane of marginal cells in the stria vascularis include discontinuities on the fracture faces suggesting membrane fusion (Forge and Fradis, 1985) and unusual convolutions of the membrane which are also seen in lipid bodies developing inside marginal cells (Forge, unpublished). Since these changes were found following chronic treatment, it needs to be established whether they relate to an initial direct effect of the drug on the cells or to subsequent pathological changes following the toxic actions in the tissues.

In summary, this work has demonstrated that gentamicin can induce a visible disorganisation of

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**Fig. 9.** PS: DSPC (1:1) with 1 mM gentamicin. The liposomes have aggregated to produce two-dimensionally extensive sheet-like forms indicating fusion. Mag. ×45500; Bar = 0.2 \( \mu \)m.

**Fig. 10.** PS: DSPC (1:1) with 0.1 mM gentamicin. The extent of aggregation is markedly less pronounced than with the higher gentamicin concentration. Mag. ×18000; Bar = 0.5 \( \mu \)m.

**Fig. 11.** PIP:\textsubscript{2} PC (1:1) with 1 mM gentamicin. Small vesicle-like clumps are apparent within the aggregate. Mag. ×45000; Bar = 0.2 \( \mu \)m.

**Fig. 12.** PIP:\textsubscript{2} PC (1:1) with 1 mM gentamicin. Large numbers of small discontinuous fracture faces are exposed. In some areas (arrowed) the exposed surface shows a series of parallel lines, reminiscent of the freeze-fracture appearance of a hexagonal phase structure. Mag. ×54000; Bar = 0.2 \( \mu \)m.

**Fig. 13.** PIP:\textsubscript{2} DSPC (1:1) with 1 mM gentamicin. Large numbers of small regular structures rather than obvious fracture steps are exposed. The presence of these structures suggests disorganisation of the bilayer. Mag. ×45000; Bar = 0.2 \( \mu \)m.
the structure of membranes containing PIP₂. If, as has been hypothesised (Schacht, 1986), the interaction of aminoglycosides with this phospholipid is a key step in the sequence of events leading to drug-induced hair cell death, such membrane disruption would likely affect cell permeability, perhaps dissipating ionic gradients, permitting calcium flux into the cell or even enabling the drug to enter the cell. It is now necessary to examine in detail the structural effects of the early interaction of aminoglycosides with the organ of Corti.

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Fig. 14. PS: DSPC (1:1) with 1 mM gentamicin and 1 mM Ca²⁺. Although liposomes are aggregated, individual liposomes are separate and the extended sheet-like form is not seen. Mag. x11300; Bar = 0.5 µm.

Fig. 15. PIP₂: DSPC (1:1) with 1 mM gentamicin and 1 mM Ca²⁺. The liposomes are not only extensively aggregated but also the small structures, suggestive of bilayer disruption, are numerous (arrows). Mag. x27000; Bar = 0.25 µm.

Fig. 16. PIP₂: DSPC (1:1) with 0.1 mM gentamicin and 1 mM Ca²⁺. Again the regular, small structures are present within liposome aggregates. Mag. x90000; Bar = 0.1 µm.