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Aminoglycoside antibiotics preferentially increase permeability in phosphoinositide-containing membranes: a study with carboxyfluorescein in liposomes

Stacley Au [a], Norman D. Weiner [a] and Jochen Schacht [b]

[a] *College of Pharmacy and* [b] *Kresge Hearing Research Institute, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)*

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The rate of release from multilamellar liposomes of the fluoroscent probe carboxyfluorescein was determined as a measure of membrane permeability. Liposomes of phosphatidylcholine and different anionic phospholipids were incubated with low (1 μM) and high (3 mM) concentrations of calcium in the absence or presence of aminoglycoside antibiotics. The leakage of carboxyfluorescein into the medium was not caused by liposomal fusion as no vesicle fusion was observed in experiments with terbium and dipicolinic acid-loaded liposomes. The basal rate of carboxyfluorescein release (in the absence or presence of 1 μM calcium) from all types of liposomes ranged from 0.1 to 0.3% of trapped carboxyfluorescein per hour. The presence of 3 mM calcium caused the greatest increase in the rate of carboxyfluorescein release (about 9-fold) in liposomes containing phosphatidylinositol 4,5-bisphosphate (PIP_2) whereas liposomes containing the other anionic phospholipids (phosphatidylserine, phosphatidylinositol and phosphatidylinositol 4-phosphate) showed an approximate 5-fold increase. In the presence of 1 μM calcium, the aminoglycosides neomycin and gentamicin also increased the rate of carboxyfluorescein release, with PIP_2-containing liposomes showing a 3–5-times greater response than the other liposomes, releasing up to 4.6% of trapped carboxyfluorescein per hour. This drug-induced release was dose-dependent and antagonized by calcium. In the presence of 3 mM calcium, 0.1 mM gentamicin or neomycin were ineffective while the drug at 1 mM affected carboxyfluorescein release from PIP_2-liposomes only. The aminoglycoside antibiotics, neomycin, gentamicin, tobramycin, kanamycin, amikacin, netilmicin, as well as neamine and spectinomycin (all at 0.1 mM) showed a graded effect on the rate of carboxyfluorescein release from PIP_2-containing vesicles in the presence of 0.1 mM calcium. The magnitude of the effect correlated well with the ototoxicity of the drugs previously determined directly in cochlear perfusions in the guinea pig. The study demonstrates that aminoglycoside antibiotics are capable of altering membrane permeabilities and that this effect is most pronounced if PIP_2 is present in the bilayers. The excellent correlation between this membrane action and the in-situ toxicity of the drugs further establishes the specific role of PIP_2 in the molecular mechanism of aminoglycoside-induced hearing loss. Moreover, it confirms the usefulness of such physicochemical models for the screening and prediction of aminoglycoside toxicity.

Introduction

Interactions of aminoglycoside antibiotics with components of the cell membrane are well documented as part of their toxic actions. Competition with calcium underlies the acute neuromuscular blockade [1] as well as the acute depression of the microphonic potential of the inner ear [2].

Correspondence: N.D. Weiner.

Antagonism with calcium and magnesium has also been reported for these drugs at the bacterial plasma membrane [3].

In addition to this displacement of calcium from membrane binding sites, specific interactions of the aminoglycoside with the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) have been documented. The drugs inhibit the metabolism of the lipid both in vitro [4] and in vivo in the tissues which are susceptible to their adverse toxic actions, i.e., inner ear [5] and kidney [6]. The binding of the aminoglycosides to PIP_2 which underlies the metabolic effects shows a number of unique physicochemical characteristics that are not seen with other phospholipids. The aminoglycoside–PIP_2 interaction creates an area-determining complex in lipid monolayers [7]; it raises the transition temperature [8] and causes phase separation of lipid components in liposomes [9]. Since the magnitude of these effects correlates well with the intrinsic ototoxicity of a series of aminoglycoside antibiotics, we have suggested that this interaction is a crucial step in determining toxicity of these drugs [7,10].

While the earlier studies clearly demonstrated alterations of membrane structure as a result of aminoglycoside-PIP_2 binding, the potential consequences for the integrity of the membrane bilayer remained to be established. The effect of drugs on the integrity of membranes is conveniently measured in lipid vesicles with the water-soluble fluorescent marker carboxyfluorescein. Its fluorescence is self-quenched during containment in liposomes and leakge into the surrounding medium can be continuously and sensitively monitored as an increase in fluorescence due to the dilution of the marker [11]. The present study examines the influence of aminoglycosides on the rate of efflux of carboxyfluorescein from liposomes of different composition.

Materials and Methods

L-α-Phosphatidylcholine (PC, from frozen egg yolk), L-α-phosphatidylserine (PS), L-α-phosphatidylinositol (PI), neomycin sulfate, spectinomycin dihydrochloride, dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), sodium citrate, Triton X-100 and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co., St. Louis, MO; terbium chloride ($TbCl_3$, 99.9% pure) from Alfa Products, Danvers, MS; Sephadex LH-20 from Pharmacia, Piscataway, NJ. Gentamicin sulfate and netilmicin sulfate were supplied by the Schering Corp. (Bloomfield, NJ); amikacin and kanamycin by Bristol Laboratories (New York, NY); tobramycin from the Eli Lilly Company, Indianapolis, IN; and neamine from the Upjohn Company, Kalamazoo, MI. Phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) were isolated from Sigma brain extracts (Cat. No. P6023) by chromatography on immobilized neomycin [12]. Lipids were stored in the dark under nitrogen at $-20\,^{\circ}C$; PIP and PIP_2 were dried and the other lipids were kept dissolved in chloroform. The purity of the lipids was greater than 98% as estimated by thin-layer chromatography (TLC). The water was thrice distilled, the second and third time in an all-glass apparatus. Crude carboxyfluorescein (Eastman Kodak, Rochester, NY) was purified by the method described by Ralston et al. [13]. The resultant carboxyfluorescein showed no impurities by TLC. The buffer for all experiments was 0.05 M Hepes (*N*-2-hydroxyethyl-1-piperazine-*N'*-2-ethanesulfonic acid), pH 7.0; ionic strength 0.2, adjusted with sodium chloride. All other chemicals were of reagent grade.

Preparation of liposomes. Chloroform solutions of the lipid mixtures contained in a 10-ml round-bottom flask were dried under nitrogen while the flask was rotated by hand to aid the formation of a uniformly thin lipid film at its bottom. Residual solvent was removed by storing the flask overnight under vacuum. The dried film was then suspended in buffer containing 100 mM carboxyfluorescein and the dispersion was vortexed intermittently for one hour at room temperature. The resultant phospholipid concentration was about 30 mM. Free carboxyfluorescein was removed by passage of the dispersion through a 1×45 cm column of Sephadex G-75 where the vesicles eluted with the void volume.

Carboxyfluorescein studies. Aliquots of the liposomal stock preparations (diluted to about 5 mM) were incubated with various concentrations of calcium chloride or aminoglycosides or both at room temperature and fluorescence was recorded

continuously for 8–10 hours. For experiments carried out in the absence of calcium, the incubation medium contained one mM EDTA. At the end of each experiment, total carboxyfluorescein fluorescence was determined after lysing the liposomes with 0.1% Triton X-100 and mixing the sample vigorously for 5 min at room temperature.

Fluorescence measurements were made with a Perkin-Elmer, Model LS-5 spectrofluorometer (excitation and emission slit widths were 10 nm and 3 nm, respectively). Carboxyfluorescein was excited at 490 nm and emission was read at 520 nm. All experiments were done in triplicate.

Terbium-dipicolinic acid studies. The terbium-dipicolinic acid assay was based on the method described by Wilschut et al. [14]. Three populations of multilamellar liposomes were prepared as described above from PC and an anionic phospholipid (PS or PIP_2) at a molar ratio 4 : 1. The vesicles contained the following aqueous media in place of carboxyfluorescein: (I) 2.5 mM $TbCl_3$ and 50 mM sodium citrate; (II) 50 mM DPA; and (III) 2.5 mM $TbCl_3$, 50 mM DPA and 50 mM sodium citrate. Vesicles were separated from nonencapsulated material by passage of the dispersion through a 1 × 45 cm column of Sephadex G-75. One mM EDTA was included in the elution buffer for all the liposome preparations.

Mixtures (1 : 1, v/v) of liposome preparations (I) and (II) were incubated with 1 mM calcium chloride or gentamicin (0.1 mM or 1.0 mM) or both in buffer at room temperature and fluorescence measurements were carried out at various time intervals. The slit width was 10 nm for both excitation (275 nm) and emission (545 nm). Maximum Tb fluorescence (that obtained upon complete fusion of the two liposome populations) was determined by measuring the fluorescence intensity of a quantity of liposome preparation (III) equal to that of the mixture of preparation (I) and (II). All experiments were done in triplicate.

Results

The rates of carboxyfluorescein (CF) leakage are expressed as per cent of total trapped CF released

$$\%CF_{released} = \left(\frac{F - F_0}{F_t - F_0}\right) \times 100 \quad (1)$$

where F is the fluorescence intensity measured at a specified time, F_0 at zero time, and F_t the total fluorescence after Triton disruption. F_t is corrected for the dilution introduced by the addition of Triton. Incubation of the liposomes with higher concentrations of Triton X-100 (1%) and at higher temperatures (60 °C) did not affect the value of F_t indicating that the procedure resulted in a complete release of dye from the liposomes.

In order to determine the potential contribution of vesicle fusion on the leakage of carboxyfluorescein, liposomes of PC, PC/PS or PC/PIP_2 (molar ratio, 4 : 1) containing $TbCl_3$ were in-

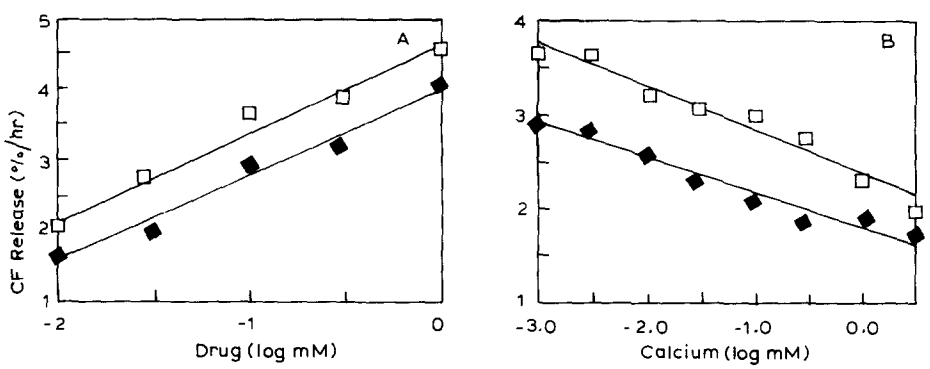

Fig. 1. Effects of drugs and calcium on carboxyfluorescein (CF) leakage from PIP_2-containing liposomes. Aliquots of the liposomal stock preparations were incubated as described in Methods. (A) Effect of neomycin (□) and gentamicin (◆) concentration on the rate of carboxyfluorescein release from liposomes in the presence of 1 μM calcium. (B) Effect of calcium concentration on the rate of carboxyfluorescein release from liposomes in the presence of 0.1 mM neomycin (□) or gentamicin (◆).

TABLE I

EFFECTS OF CALCIUM AND AMINOGLYCOSIDES ON RELEASE OF CARBOXYFLUORESCEIN

Carboxyfluorescein release (% leakage/h) from mixed liposomes of PC plus the anionic lipid indicated (molar ratio, 4:1) was determined as described in Methods.

Anionic lipid	Neomycin (mM)	Gentamycin (mM)	Release at	
			0 and 1 μM Ca^{2+}	3 mM Ca^{2+}
PS	0	0	0.2	0.96
PS	0.1	0	1.07	0.89
PS	1.0	0	1.16	0.89
PS	0	0.1	0.70	0.89
PS	0	1.0	0.90	0.89
PI	0	0	0.2	0.96
PI	0.1	0	1.10	0.96
PI	1.0	0	1.16	0.96
PI	0	0.1	0.66	0.96
PI	0	1.0	0.90	0.96
PIP	0	0	0.2	1.10
PIP	0.1	0	1.22	1.08
PIP	1.0	0	1.27	1.08
PIP	0	0.1	0.90	1.08
PIP	0	1.0	1.00	1.08
PIP_2	0	0	0.2	1.71
PIP_2	0.1	0	3.62	2.02
PIP_2	1.0	0	4.61	2.67
PIP_2	0	0.1	2.91	1.75
PIP_2	0	1.0	4.08	2.06

cubated with an equal aliquot containing dipicolinic acid. In the absence of cations, fluorescence intensity remained constant for at least 3 hours. Addition of calcium or gentamicin or both to any of the liposomes did not change the Tb fluorescence significantly. Less than 0.25% of the maximal fluorescence expected from liposome fusion was detected under these conditions.

Preliminary experiments had indicated that liposomes at molar ratios of PC/anionic lipid of 2:1 or 1:1 were very leaky to carboxyfluorescein resulting in high basal release values interfering with the determination of drug-induced changes. In contrast, leakage at molar ratios of 4:1 was minimal, the rate of carboxyfluorescein release from all liposomes tested ranged from 0.1 to 0.3% of trapped carboxyfluorescein per hour was essentially linear ($r \geq 0.98$) over 6 to 8 hours, i.e., the entire time course of the experiments. For liposomes of all compositions, the release rates were identical whether calcium was absent or present at 1 μM. This included the release rates in the additional presence of aminoglycosides.

The addition of 3 mM calcium resulted in significant increases of carboxyfluorescein release in all liposomes and particularly in PIP_2-containing liposomes which showed an approximate 8-fold increase (Table I). The different behavior of PIP_2-

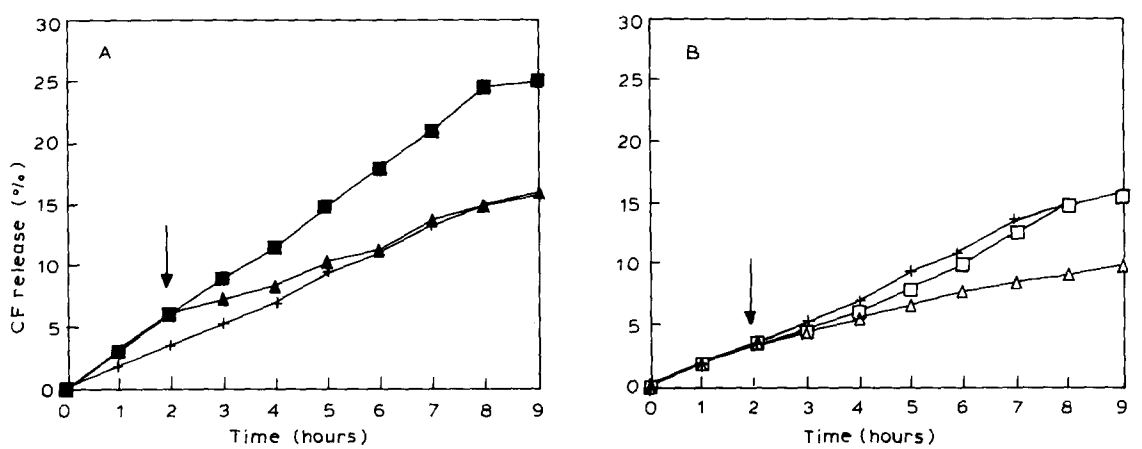

Fig. 2. Reversibility of the calcium–aminoglycoside interaction. Changes in the rate of carboxyfluorescein (CF) release upon sequential addition of calcium and drug were obtained as described in Methods. (A) Effect of 1 mM calcium on the rate of carboxyfluorescein release from PIP_2-containing liposomes in the presence of 0.1 mM gentamicin. Key: (■) gentamicin alone, no calcium added; (+) gentamicin and calcium added at time zero; (▲) gentamicin added at time zero, calcium at $t = 2$ h (arrow). (B) Effect of 0.1 mM gentamicin on the rate of carboxyfluorescein release from PIP_2-containing liposomes in the presence of 1 mM calcium. Key: (△) calcium alone, no gentamicin added; (+) calcium and gentamicin added at time zero; (□) calcium added at time zero, gentamicin at $t = 2$ h (arrow).

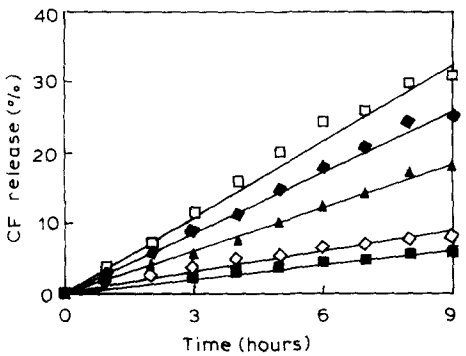

Fig. 3. Effect of aminoglycoside antibiotics on carboxyfluorescein (CF) leakage from PIP_2-containing liposomes. Aliquots of the liposomal stock preparations were incubated as described in Methods with 0.1 mM drug in the presence of 1 μM calcium. Key: (□) neomycin; (◆) gentamicin and tobramycin (represented as a single curve for the sake of clarity); (▲) kanamycin, amikacin and netilmicin (represented as a single curve for the sake of clarity); (◇) neamine; (■) spectinomycin.

containing liposomes was also evident when liposomes were challenged with neomycin or gentamicin. The addition of 0.1 mM aminoglycoside was about as effective as 3 mM calcium across all liposomes in inducing carboxyfluorescein leakage and the aminoglycoside was again most active against PIP_2 vesicles. The rates were further increased if the aminoglycoside concentration was raised to 1 mM, and neomycin was more effective than gentamycin. For PIP_2 vesicles, the release rate was increased approximately 20-fold in the presence of 1 mM neomycin over that of the controls.

In the presence of 1 μM calcium, the rate of carboxyfluorescein release from PIP_2 liposomes by neomycin or gentamicin was dose-dependent with neomycin somewhat more effective than gentamicin (Fig. 1A). Both drugs significantly increased carboxyfluorescein release even at a concentration of 10 μM. The competitive effect of calcium was also dose-dependent (Fig. 1B), and 10 μM calcium was the lowest effective concentration to antagonize 0.1 mM drug.

The effects of calcium and of aminoglycosides were not additive but competitive (Table I). This is indicated by the combinations of 0.1 mM and 1 mM neomycin or gentamicin with 3 mM calcium. For example, 1 mM neomycin alone (or in the presence of 1 μM calcium) induced a higher rate of release in all liposomes than 3 mM calcium alone. Upon combined drug/calcium challenge, the rate was not different from that of calcium alone except for PIP_2-containing liposomes. In the latter case, an intermediate rate was observed.

The reversibility of the calcium and aminoglycoside actions was indicated by the changes in the rate of carboxyfluorescein release upon sequential addition of calcium and drug (Fig. 2). When 1 mM calcium was added after two hours to an incubation proceeding with 0.1 mM gentamicin, the rate of carboxyfluorescein release was lowered. Conversely, when 0.1 mM gentamicin was added after two hours to an incubation proceeding with 1 mM calcium, The rate was increased.

A comparison of different aminoglycosides (at 0.1 mM) in the presence of 1 μM calcium on the rate of carboxyfluorescein release (Fig. 3) demonstrated three apparent levels of effectiveness: Neomycin had the greatest influence on the carboxyfluorescein release, (3.6%/h); gentamicin and tobramycin each released 2.9%/h; kanamycin, amikacin, and netilmicin did not act significantly different from each other, each releasing around 2.1%/h. The neomycin fragment neamine and the aminocyclitol antibiotic spectinomycin caused a release slightly above background, 0.9%/h and 0.6%/h, respectively.

Discussion

The increase in the rate of carboxyfluorescein leakage induced by drugs and calcium can be the result of at least three processes: (1) an 'all-or-none' rupture of the permeability barrier of a portion of the vesicles; (2) cation-induced fusion of the liposomes; or (3) alteration of the barrier properties of the bilayer leading to an increased permeability coefficient of the dye through the bilayer. The first mechanism, although theoretically possible if the number of vesicles ruptured per unit time is small, seems unlikely since rupture can be expected to occur within a short period of time [15]. In these experiments, both in the absence and presence of cations, the leakage is slow (0.2–5%/h) and, more importantly, the rate of carboxyfluorescein release remains essentially constant for at least 8 hours. The second mechanism

is not supported by the Tb-DPA studies. This sensitive indicator rules out any significant fusion under the experimental conditions. Although polyvalent cations have been shown to induce liposome fusion, particularly with small unilamellar vesicles [14], our results are in good agreement with studies showing that liposomes with more than 50 mol% PC are not fused by cations such as calcium [16]. Therefore, the increase in the rate of carboxyfluorescein leakage induced by calcium and the aminoglycosides is a consequence of the cations' ability to alter the permeability of the bilayer to the dye molecule.

The ability of the aminoglycosides to affect the leakage of the water-soluble indicator depends on a number of factors: (i) the lipid composition of the liposome; (ii) the concentration of calcium present; and (iii) the structure and concentration of the aminoglycoside. With respect to the effect of lipid composition of the liposome, it is quite remarkable that PS, PI and PIP all react alike despite differences in structure and charge. In contrast to this stands the behavior of PIP_2-containing liposomes which were significantly more interactive with aminoglycosides under all conditions. It should be pointed out that for PIP_2 liposomes, drug-induced leakiness can be observed at antibiotic concentrations as low as 10 μM (Fig. 1A).

The uniqueness of PIP_2, which was most striking at the higher calcium concentration because no other lipid responded to the aminoglycosides, is in good agreement with previous studies of aminoglycoside–lipid interactions. Recently, Au et al. [9] reported that liposomes containing PIP_2 showed the greatest aminoglycoside-induced membrane perturbation as measured by changes in 1-anilino-8-naphthalene sulfonate (ANS) fluorescence when compared to liposomes containing other anionic phospholipids (PS, PI or PIP). Neomycin also lowered the phase transition temperature of PIP_2-PC liposomes while it raised the transition temperature of all other anionic phospholipid liposomes tested [8].

With respect to the influence of calcium on aminoglycoside induced leakage, a number of points deserve attention. Firstly, carboxyfluorescein release in the presence of aminoglycosides was unaffected by the presence of 1 μM calcium. This concentration of calcium approximates biological intracellular levels and was considered appropriate for comparative studies since PIP_2 is believed to reside in the inner bilayers of cytoplasmic membranes [17]. Thus, if aminoglycosides are transported into susceptible tissues by an active uptake system as described for the kidney and the ear [18], the aminoglycosides will compete with low concentrations of calcium. Although the aminoglycoside actions are reversible with calcium (Fig. 2), the equilibrium will favor the drug/lipid binding at intracellular conditions. These conclusions are also supported by studies [9] showing that membrane disturbance by aminoglycoside antibiotics as investigated in liposomes containing the fluorescent probe, ANS, was unaffected by the presence of 1 μM calcium. Since the rate of leakage is increased by about one order of magnitude even at these low drug concentrations, biological membranes containing this lipid should be quite susceptible to damage by aminoglycosides.

Recently, we presented a multi-step hypothesis of aminoglycoside ototoxicity [19,20] which is consistent with the data elaborated by our laboratory and others: (1) The first step in the proposed reaction sequence is an electrostatic interaction of the aminoglycoside with negatively charged components of the outer plasma membrane. The resulting dispalcement of calcium accounts for the acute effects of the drug action and is reversible and antagonized by cations. (2) The drug is then transported into the cell by an energy-dependent process. (3) The next and most crucial step is the binding of the drug to phosphatidylinositol bisphosphate. The formation of the drug-lipid complex prevents the hydrolysis of PIP_2 and thereby interferes with its physiological role in the phosphoinositide second messenger system. Furthermore, it disturbs membrane integrity and structure. The data presented in this study show that binding of the drug to PIP_2 can in fact result in permeability changes of the membrane. Such increased leakiness in a biological membrane may well be a critical factor leading to cell death.

The excellent correlation (Fig. 4) between the ability of different aminoglycosides to affect the rate of carboxyfluorescein release from PC : PIP_2 liposomes (molar ratio 4 : 1) and their ototoxicity as determined by chochlear perfusion [7,10] sup-

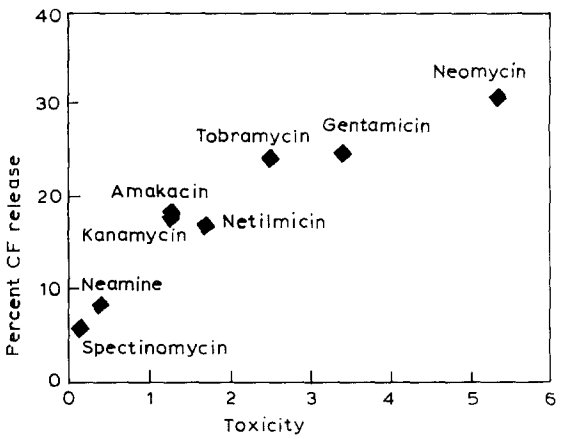

Fig. 4. Correlation of the effect of aminoglycosides on carboxyfluorescein (CF) release (shown in Fig. 3) with ototoxicity. Ordinate: percentage of carboxyfluorescein release from liposomes after incubation with 0.1 mM aminoglycosides for 9 hours. Abscissa: ototoxicity of various aminoglycosides as determined by chochlear perfusions (see Ref. 10). 1, Spectinomycin; 2, neamine; 3, kanamycin; 4, amikacin; 5, netilmicin; 6, tobramycin; 7, gentamycin; 8, neomycin.

ports the contention that drug binding to PIP_2 is an integral and decisive step in the mechanism of aminoglycoside toxicity. This correlation was similar to that reported for the effects of aminoglycosides on the fluorescence intensity of ANS-containing liposomes and ototoxicity [10]. The remarkable similarity between the two results (ANS probes structural changes and carboxyfluorescein probes the resultant permeability changes induced by the drug) suggests that the drug–PIP_2 interaction is a quantitative indicator of toxicity and may thus be used in in vitro ototoxicity testing.

Acknowledgement

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