

BINDING OF NEOMYCIN AND CALCIUM TO PHOSPHOLIPIDS
AND OTHER ANIONIC COMPOUNDS

SALLY E. WILLIAMS and JOCHEN SCHACHT*

Kresge Hearing Research Institute, The University of Michigan,
Ann Arbor, Michigan 48109, U.S.A.

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In order to assess the potential importance of different cellular binding sites for the adverse effects of aminoglycosides (*i.e.* oto- and nephrotoxicity) the binding of neomycin and calcium to phospholipids and other anionic cell constituents was assayed *in vitro*. Phospholipids demonstrated binding affinities that strongly favored neomycin (K_m , 10 to 100 μM) over calcium (K_m , 300 to 1,120 μM). Both neomycin and calcium showed strongest binding to lipids with monoester phosphate groups: phosphatidic acid, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate. The lipids bound 0.2 to 0.4 molecules of neomycin and 0.5 to 1 molecule of calcium, respectively, per lipid. Anionic non-lipid compounds such as melanin, gangliosides or chondroitin sulfate were ineffective competitors of neomycin binding to lipids.

The results emphasize the importance of phospholipids as cellular binding sites for aminoglycosides. Furthermore, if one considers extra- and intracellular calcium and neomycin concentrations, the relative affinity of lipids for these two compounds suggests an explanation for both the reversible and the essentially irreversible toxic effects of the aminoglycosides.

The ototoxicity of aminoglycoside antibiotics is well documented and a rational hypothesis of their action has been proposed.^{1,2)} However, some details of this mechanism have yet to be fully described. There is evidence that a major component of the actions of these drugs is exerted at the cell membrane. Acute effects on the microphonic potential of the cochlea³⁾ and the lateral line organ⁴⁾ are rapidly reversed by the addition of competing cations indicative of a plasma membrane action. Chronic toxicity has also been linked to an interference with the metabolism and function of membrane lipids, in particular, of the polyphosphoinositides. For example, the aminoglycoside neomycin, has been shown to inhibit labeling of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) *in vivo* and to prevent enzymatic hydrolysis of polyphosphoinositides *in vitro*.^{5,6)} Furthermore, a strong correlation exists between the degree of ototoxicity of the aminoglycosides and their ability to disrupt the structure of artificial phosphoinositide membranes.^{7,8)}

While the polyphosphoinositides appear to be a major intracellular site of aminoglycoside action, these drugs also bind to other lipids and anionic cell components.^{8~11)} The contribution of these interactions to acute and chronic toxicity has not yet been systematically assessed. This study aims to determine the relative binding affinity of neomycin and calcium, the major cation of excitable membranes, for various phospholipids and other macromolecules in order to gain more insight into potential cellular binding sites for aminoglycosides.

Materials and Methods

Phosphatidyl serine (PtdSer), phosphatidylinositol (PtdIns), phosphatidic acid (Ptd), phosphatidyl glycerol (PtdGro) and cardiolipin were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) were

isolated from Sigma phosphoinositides (catalogue no. P6023) by chromatography on immobilized neomycin.¹²⁾ The purity of the lipids was greater than 95% as determined by thin-layer chromatography.

Radioactive calcium was purchased from NEN, Boston, MA. [¹⁴C]Neomycin was a gift from Dr. GORDON FLYNN, The University of Michigan.

Methods

Lipid-bound calcium and neomycin were determined in a biphasic chloroform-water system with a final volume of 0.20 ml in each phase. In both assays the lipid phase contained 0.1 mM lipid in chloroform. Lipid concentrations were determined by assay of lipid phosphorus.¹³⁾

Calcium Binding: The aqueous phase contained 187 mM NaCl, 10 mM sodium *N*-2-hydroxyethylpiperazine *N*-2-ethanesulfonic acid (HEPES), pH 7.4, and ⁴⁵Ca⁺⁺ in concentrations of 0.1 to 1 mM (0.1 μ Ci).

Neomycin Binding: The aqueous phase contained [¹⁴C]neomycin in NaCl-HEPES as above to a final concentration of 0.1 to 1 mM (specific radioactivity 1.35 mCi/mmol).

The phases were mixed for 30 seconds and separated by centrifugation for 15 minutes at 2,000 rpm. The aqueous layer was removed and radioactivity in the lipid phase, indicative of bound calcium or neomycin, was determined by liquid scintillation counting. Blanks contained all components of the assay except the lipid. Radioactivity in these blanks did not significantly exceed background counts.

Competition Studies: CaCl₂ was added to the [¹⁴C]neomycin-containing aqueous phase to a final concentration of 1 to 10 mM. Conversely, 0.1 to 1 mM neomycin were added to the ⁴⁵Ca-assays.

Displacement Studies: [¹⁴C]Neomycin was bound to lipid as described above and the lipid layer was used in the displacement assay. The lipid phase was repartitioned with 200 μ l of a newly added aqueous phase containing 1 to 10 mM of the competitor in NaCl-HEPES buffer. The phases were mixed, centrifuged and aliquots were taken from the aqueous layer for determination of displaced [¹⁴C]neomycin.

Calculations: Michaelis-Menton constants (*Km*) were calculated either from Lineweaver-Burke or Scatchard plots based on least squares analysis. The number of binding sites was quantitated from Scatchard plots.¹⁴⁾

Results

Neomycin Binding

Neomycin bound strongly to all of the phospholipids tested with binding affinities (expressed as *Km*) in the range of 10 to 100 μ M (Table 1).

Table 1. Neomycin binding to phospholipids.

Lipids	<i>Km</i> (μ M)	<i>n</i> (Neomycin bound/lipid)
PtdIns	86 \pm 13	0.25 \pm 0.04
PtdSer	43 \pm 18	0.18 \pm 0.02
Ptd	10 \pm 4	0.39 \pm 0.001
PtdInsP	(I) 23 \pm 17	0.15 \pm 0.02
	(II) 250 \pm 56	0.43 \pm 0.08
PtdInsP ₂	(I) 11 \pm 4	0.19 \pm 0.05
	(II) 46 \pm 11	0.36 \pm 0.07

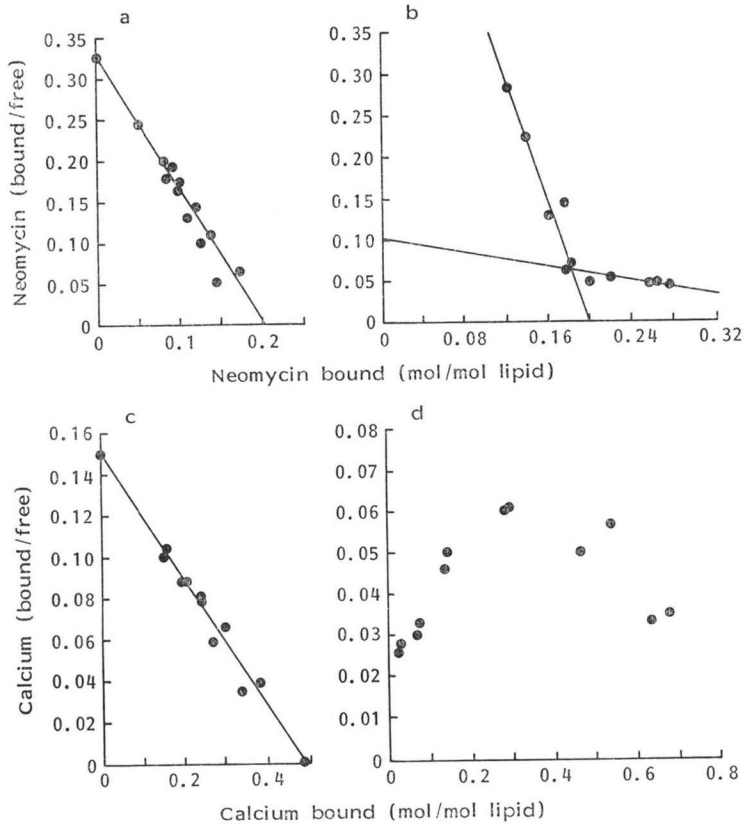
Binding of neomycin to lipids was analyzed as described in "Methods." Values are means \pm standard deviations of 3 to 6 individual experiments; in each experiment, *Km* and *n* were determined from a series of 5 concentrations of neomycin, each concentration tested in duplicate.

The lipids Ptd, PtdInsP and PtdInsP₂ displayed the strongest affinities (*Km*=10 μ M, 23 μ M, and 11 μ M respectively), followed by PtdSer and PtdIns (*Km*=43 μ M and 86 μ M). Scatchard analysis indicated a single binding site for Ptd, PtdSer and PtdIns and two binding sites for PtdInsP and PtdInsP₂ (Figs. 1a and 1b show representative graphs). Binding site "I" of PtdInsP and PtdInsP₂ had an affinity in the same range as Ptd, PtdSer and PtdIns. The second binding site ("II") of PtdInsP₂ also showed a relatively high affinity (*Km*=46 μ M), but site II of PtdInsP displayed a 10-fold lower affinity than

Fig. 1. Neomycin and calcium binding to phospholipids.

Binding experiments were conducted as described in Materials and Methods. Data is presented in Scatchard plots, each from a representative experiment.

a) Neomycin binding to PtdIns; also representative of Ptd and PtdSer. b) Neomycin binding to PtdInsP₂; also representative of PtdInsP. c) Calcium binding to PtdInsP; representative for all lipids tested except PtdInsP₂. d) Calcium binding to PtdInsP₂.



its site "I" ($K_m=250 \mu\text{M}$).

The lipids tested bound from 0.15 to 0.43 neomycin molecules per lipid molecule, *i.e.* one neomycin molecule bound to two to five lipid molecules. Ptd and binding site "II" of PtdInsP and PtdInsP₂ bound approximately twice as much neomycin (0.4 neomycin per lipid) as did PtdSer or their respective sites "I" (0.2 neomycin per lipid).

Calcium Binding

As with neomycin, the lipids Ptd, PtdInsP and PtdInsP₂ demonstrated the strongest affinity for calcium, followed by PtdSer, cardiolipin and PtdIns (Table 2). However, the affinities ranged from 300 to 1,118 μM , indicating as much as a 30 to 45-fold lower affinity of individual lipids for calcium than for neomycin. All the lipids tested exhibited a single binding site upon Scatchard analysis (example, Fig. 1c) except PtdInsP₂ which displayed a parabolic curve (Fig. 1d) usually seen in systems with positive cooperativity. Analysis of the Hill coefficient,¹⁴ however, did not support this interpretation ($n=1.17 \pm 0.85$).

Table 2. Calcium binding to phospholipids.

Lipids	Km (μM)	n (Calcium bound/lipid)
PtdIns	1,118 \pm 315	0.43 \pm 0.17
PtdSer	600 \pm 80	0.41 \pm 0.13
Ptd	300 \pm 30	0.54 \pm 0.08
PtdGro	800 \pm 240	0.54 \pm 0.05
Cardiolipin	600 \pm 60	0.81 \pm 0.28
PtdInsP	360 \pm 168	0.95 \pm 0.16
PtdInsP ₂	450 \pm 194	0.99 \pm 0.17

Binding of calcium to lipids was analyzed as described in "Methods." Values are means \pm standard deviations of 5 to 13 individual experiments; in each experiment, Km and n were determined from a series of 5 concentrations of Ca^{++} , each concentration tested in duplicate. Values for PtdInsP₂ were calculated from the linear negative slope of the Scatchard analysis (see Fig. 1d).

The number of calcium molecules bound per lipid ranged from 0.4 to 0.5 (*i.e.* approximately one calcium per two lipid molecules) for PtdIns, PtdSer, Ptd and PtdGro to approximately one calcium molecule per lipid for PtdInsP, PtdInsP₂ and cardiolipin.

Competition Between Neomycin and Calcium for Lipid Binding Sites

Neomycin and calcium acted as mutual inhibitors of their binding. The binding of neomycin to lipids was competitively inhibited by Ca^{++} with inhibitor constants ranging from 300 to 900 μM . They were for PtdSer, 430 \pm 60 μM ; for PtdIns, 360 \pm 110 μM ; for PtdInsP site I, 510 \pm 130 μM ; site II 910 \pm 210 μM ; for PtdInsP₂ site I, 470 \pm 220 μM ; site II 360 \pm 300 μM . Conversely, neomycin was a competitive inhibitor of ⁴⁵Ca binding. The inhibitor constants with PtdIns and PtdInsP were 40 \pm 25 μM and 53 \pm 36 μM respectively.

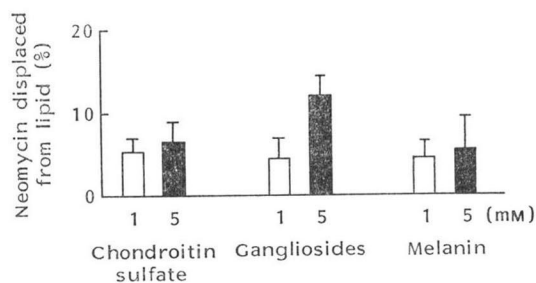
Competition Between Lipids and Non-lipid Compounds for Neomycin

The ability of anionic non-lipid compounds to compete for neomycin bound to a phospholipid was studied in "displacement" assays. The compounds tested were cellular components with a reported ability to bind aminoglycosides (melanin¹¹⁾ and the mucopolysaccharide chondroitin sulfate¹⁰⁾ or calcium (gangliosides¹⁵⁾). The assay was based on the assumption that 50% of the bound neomycin would be displaced from the lipid if the tested molecule had an equal affinity for neomycin and was added in equimolar amounts to the lipid.

Chondroitin sulfate, gangliosides and melanin all were inefficient competitors. The displacement was dose dependent, but at 1 mM, *i.e.* at a 10-fold higher concentration than the lipid, only 5% of the bound neomycin was displaced from PtdSer (Fig. 2). Even at concentrations of fifty times that of the lipid, a maximum of 13% of the neomycin was displaced.

Fig. 2. Competition for lipid-bound neomycin by anionic non-lipid compounds.

Displacement assays were carried out as described in "Methods." Values are expressed as the percentage of bound neomycin displaced from the lipid by the compound tested.



Discussion

All phospholipids tested in this study showed binding affinities up to fifty-fold stronger for neomycin than for calcium as seen in the individual binding constants (Km). This was further supported

by the fact that neomycin displayed much lower inhibitor constants for competition with calcium binding than did calcium for competition with neomycin binding. Binding affinities for both neomycin and calcium did not correlate with the number of lipid phosphate groups as Ptd, PtdInsP and PtdInsP₂ with one, two and three phosphates, respectively, had comparably high affinities. Conversely Ptd, PtdSer and PtdIns showed widely varied affinities. The determining factor for high binding affinity for both calcium and neomycin thus seems to be the presence of monoester phosphate groups. The rank-order of affinities of the various lipids for calcium measured in this biphasic system agrees well with those extrapolated from calcium adsorption to PtdInsP₂, Ptd and PtdSer in monolayers.¹⁶⁾

Although the total number of phosphate groups is not a factor in the affinity of the binding, these sites do determine the number of calcium molecules bound by the lipid. Lipids with potentially three (PtdInsP and cardiolipin) and five (PtdInsP₂) negative charges bind more calcium molecules, but this correlation did not hold for neomycin binding. Here, steric hindrance caused by the large size of the neomycin molecule may be an additional determining factor.¹⁷⁾

While all phospholipids have a high affinity for neomycin, the other anionic compounds tested were inefficient in their competition for the drug. Neither melanin, chondroitin sulfate nor gangliosides were able to displace significant amounts of neomycin from lipid. Extrapolations from the *in vitro* to the *in vivo* situation have to be made with caution but it should be emphasized that the binding data were obtained at a pH and a concentration of monovalent cations that resemble physiological conditions. Thus, the results strongly indicate that phospholipids will be preferred cellular drug binding sites and that melanin, mucopolysaccharides and gangliosides are less important in the toxic actions of aminoglycosides.

The differences in affinity of the various lipids to neomycin do not seem significant enough to explain that this drug specifically affects the metabolism of phosphoinositides *in vivo* and *in vitro*.^{1,5,6)} However, if we consider the asymmetric lipid distribution of natural membranes as well as the differences in extra- and intracellular calcium concentrations an explanation becomes apparent. Since polyphosphoinositides are thought to be present on the cytoplasmic side of the plasma membrane, they would not be the first target of the drug in its interaction with the cell. The drug would first bind to an anionic site on the outer membrane leaflet, probably PtdSer, PtdGro, or PtdIns. This binding is in competition with extracellular calcium, the concentration of which is well in the range of the *K_i* of calcium inhibition of lipid-neomycin binding. Thus, any acute drug toxicity based on calcium displacement may easily be reversed. Next, the drug is transported across the membrane in an energy-dependent process.²⁾ Once inside the cell, the drug would bind preferentially to a polyphosphoinositide or phosphatidic acid. From pharmacokinetic studies¹⁸⁾ we can estimate the intracellular aminoglycoside concentration to reach 10 to 100 μM in the inner ear tissues while the intracellular calcium concentration is in the low micromolar range. Given the fifty-fold difference in binding affinities, neomycin would effectively and essentially irreversibly displace calcium from these lipids. The resultant inhibition of polyphosphoinositide metabolism⁵⁾ and disturbance of membrane integrity would then account for further toxic effects of aminoglycosides.¹⁾ It is important to note that phosphatidylinositol 4,5-bisphosphate has recently been implicated as a physiologically important lipid constituting a second messenger system for hormone actions that regulate cell processes *via* the mobilization of intracellular calcium.^{19, 20)}

In conclusion, the results emphasize the importance of phospholipids as cellular binding sites for aminoglycosides. Furthermore, the comparison of the relative affinities for calcium and for neomycin under consideration of extra- and intracellular calcium concentrations suggest an explanation for the reversible and for the essentially irreversible effects of the antibiotics.

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