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Isolation of an Aminoglycoside Receptor from Guinea Pig Inner Ear Tissues and Kidney*

Jochen Schacht

Kresge Hearing Research Institute, The University of Michigan, Ann Arbor, MI 48109, USA

Summary. Affinity chromatography was used to isolate aminoglycoside receptors from inner ear tissues and kidney. Neomycin was immobilized on glass beads and served as the stationary phase in column chromatography. Fractionation of an organic tissue extract on this matrix demonstrated two components with high affinity for neomycin: phosphatidyl inositol phosphate and phosphatidyl inositol diphosphate. The toxicity of aminoglycosides is explained on the basis of a drug-interaction with these lipids.

Key words: Aminoglycoside ototoxicity – Neomycin – Drug receptors – Affinity chromatography – Polyphosphoinositides

The mechanism of the bactericidal action of aminoglycosides has been well established as an interference with protein synthesis (Pestka, 1971). Difference in this synthetic machinery between prokaryotic and eukaryotic cells as well as experimental evidence (Stockhorst and Schacht, 1977) make such a mechanism unlikely for the ototoxic and nephrotoxic action of these drugs.

The selective action of the aminoglycosides on inner ear and kidney may suggest the presence in these tissues of a specific drug receptor. If such an aminoglycoside receptor exists it may be a component of the plasma membrane since it is necessarily the first site of encounter with the drug. Moreover, neomycin is a basic antibiotic and should interact with acidic membrane components.

A well-established procedure to isolate membrane receptors for hormones and neurotransmitters is affinity chromatography, which exploits the specific and usually high affinity of a ligand to its membrane receptor. In this procedure, the ligand in question is chemically fixed to a stationary matrix, such as a dextrane or glass support. This immobilized ligand then serves as a specific exchanger in column chromatography. When a cell extract is passed through such a column, the appro-

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priate membrane receptor will bind and can thus be isolated. We are reporting here immobilized neomycin as an agent for affinity chromatography. Extracts of guinea pig inner ear tissues and guinea pig kidney were chromatographed over this immobilized neomycin and membrane components showing strong affinity to neomycin were isolated.

Methods

Preparation of Immobilized Neomycin. The procedure has been described in detail elsewhere (Schacht, 1978). Briefly, a porous glass bead matrix (Glycophase-CPG, Pierce, Rockford, Ill.) was oxidized to the aldehyde. Neomycin was added to form a Schiff base via one of its amino groups. Subsequent reduction with sodium borohydride established a stable covalent bond between the glass matrix and neomycin. This adduct was used as the stationary phase in column chromatography.

Labeling of Tissues and Extraction. To label kidneys radioactively, albino guinea pigs (approximately 300 g) were injected i.p. with 100 μCi of neutralized, carrier-free ^{32}P-orthophosphate in saline (Schibeci and Schacht, 1977). One hour later, the animals were killed by decapitation and the kidney was quickly excised and homogenized in 10 ml chloroform-methanol 1 : 2 (v/v) with a Polytron (Brinkman Instruments, Westbury, N.Y.). Inner ear tissues were marked radioactively by cochlear perfusion with $^{32}P_i$ in artificial perilymph, dissected and homogenized in 1 ml of chloroform-methanol 1 : 2 (v/v) (Stockhorst and Schacht, 1977).

A lipid extract of inner ear tissues or kidney was prepared by the addition of 0.6 ml of 1 M KCl and 0.6 ml of chloroform per ml of homogenate. After mixing and centrifugation, the lower phase was collected and washed once with methanol-1 N HCl 1 : 1 (v/v). An equal volume of 200 mM ammonium acetate in methanol was added to the extract which was then applied to the chromatographic column.

The column was first washed with 150 mM ammonium acetate in chloroform-methanol-water, 3 : 6 : 1 (by vol). The next fraction collected was eluted with 600 mM ammonium acetate in the same solvent. Finally, the column was eluted with chloroform-methanol-15 N ammonia, 3 : 6 : 1 (by vol.). After elution, each fraction was acidified and the lipids washed free of salt (Schacht, 1978). Aliquots were applied to thin layer chromatographic plates (Silica gel 60, EM Lab., Elmsford, N.Y.) and developed in chloroform-methanol-15 N ammonia-water 90 : 90 : 7 : 22 (by vol). Radioactive lipids on thin layer plates were located by radioautography, as shown in Figs. 1 and 2.

Results and Discussion

The organic extract prepared from inner ear tissues and kidney contained neutral and acidic lipids such as glyco- and phospholipids, as well as lipoproteins and hydrophobic proteins which were separated by thin layer chromatography. These components were visualized by reaction with iodine vapors, ninhydrin sprays, and phosphate detecting sprays. Phospholipids of the kidney and the inner ear also became rapidly and strongly labeled by ^{32}P (Fig. 1, sample 0; Fig. 2, sample 0). These radioautograms are presented here because they provide the best means of demonstrating polyphosphoinositides, which are quantitatively minor but rapidly metabolizing lipids in these tissues.

The cell extract was then applied to the column where neomycin, based on its nature as a cation, acts as an ion-exchanger. At 150 mM salt concentration, most phospholipids and otherwise detectable components of the cell extract were eluted from the neomycin column (Figs. 1 and 2, samples 1 and 2). Elution of a component

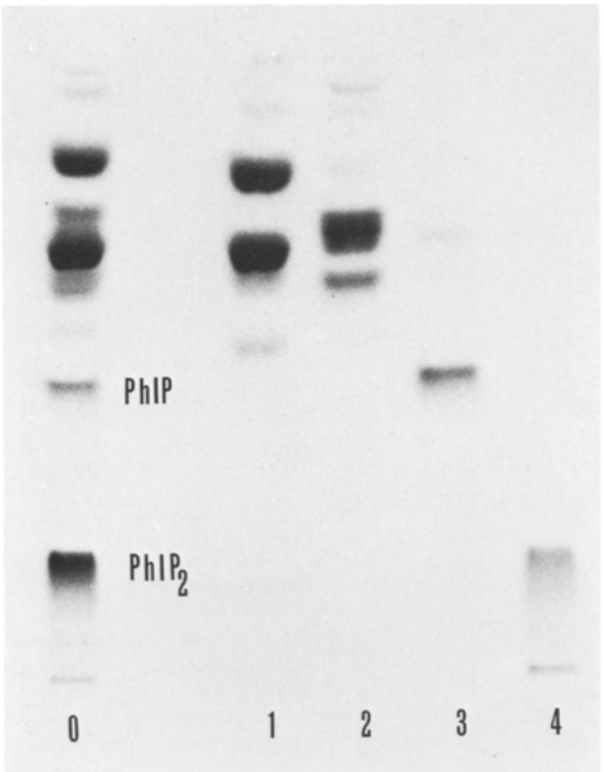

Fig. 1. Thin layer chromatography of renal lipids separated on immobilized neomycin. A total lipid extract from a guinea pig kidney, perviously labeled with $^{32}P_i$ was applied to a chromatographic column containing immobilized neomycin. Aliquots of the extract (Sample "0") and of the column fractions (1–4) were separated by thin layer chromatography and the above autoradiogram was obtained as described in "Methods". Fraction 1 was collected while the extract was applied to the column; fraction 2 was eluted with 150 mM ammonium acetate in chloroform-methanol-water 3 : 6 : 1 (by vol); fraction 3 was eluted with solvent containing 600 mM ammonium acetate, and fraction 4 with chloroform-methanol-15 N NH_4OH 3 : 6 : 1 (by vol). *Abbreviations:* PhIP, $PhIP_2$ = phosphatidyl inositol phosphate, diphosphate

at such low ionic strength suggests that it does not have a high affinity for neomycin and may not be a binding site under physiological conditions.

Only two compounds can be detected as being bound to the column after this elution: phosphatidyl inositol phosphate and phosphatidyl inositol diphosphate. The first of these two lipids can be removed from the neomycin column by increasing the salt concentration in the solvent to 600 mM (Figs. 1 and 2, sample 3). Even at such a high concentration of salt in the solvent, the second component, phosphatidyl inositol diphosphate will remain bound to neomycin. The interaction between this lipid and neomycin is so strong that the addition of hydrochloric acid or ammonia (Figs. 1 and 2, sample 4) is necessary to remove it.

This behavior of polyphosphoinositides is rather unique for the neomycin ion-exchanger and is not seen on other cationic exchangers, such as DEAE cellulose

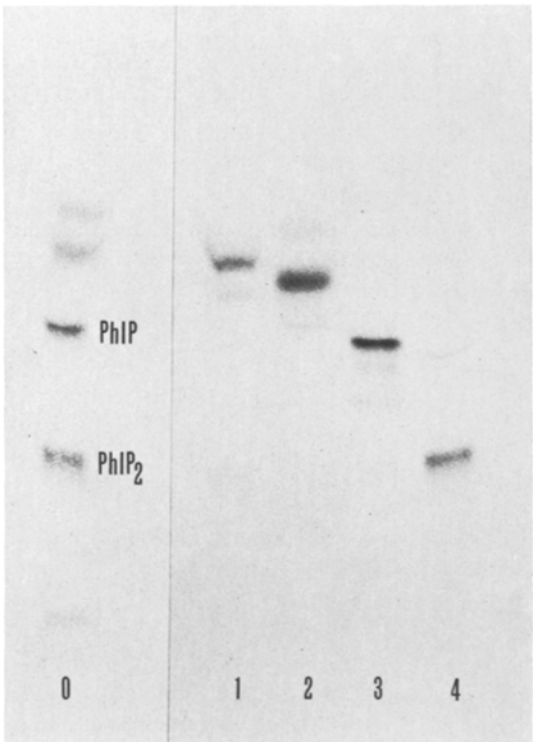

Fig. 2. Thin layer chromatography of cochlear lipids separated on immobilized neomycin. Cochlear tissues of a guinea pig were labeled with $^{32}P_i$ by perilymphatic perfusion (Stockhorst and Schacht, 1977). A total lipid extract from these tissues was separated as described in Fig. 1

(Hendrickson and Ballou, 1964). We have speculated (Schacht et al., 1977) that this highly specific interaction between neomycin and phosphatidyl inositol diphosphate is based on a stereochemical orientation that favors a triple ionic interaction between the antibiotic and the lipid. Once such a three point attachment is established, the breaking of this complex would require a simultaneous detachment at all three sites. This is statistically highly improbable and may explain the extremely tight binding between the two compounds.

It is particularly important to consider that this binding occurs at high ionic strength of the surrounding medium and even in the presence of calcium (Lodhi et al., 1976). In contrast, mucopolysaccharides which have also been suggested as binding sites for aminoglycosides have been shown not to form complexes with the drugs under physiological ionic conditions (Deguchi et al., 1978).

Extrapolations from in vitro experiments to an in vivo situation should be made with great caution. In this case, however, our in vivo experiments are consistent with the notion that a direct and highly specific interaction occurs between the polyphosphoinositides and the aminoglycosides. After chronic treatment of guinea pigs with neomycin, inhibition of polyphosphoinositide metabolism was observed in the kidney (Schibeci and Schacht, 1977) and in tissues of the inner ear (Orsulakova et al., 1976). Acute experiments with perilymphatic perfusions demonstrated a correlation between the loss of cochlear microphonics and the inhibition of polyphosphoinositide metabolism by neomycin (Stockhorst and Schacht, 1977). Such an action on

polyphosphoinositides seemed to be a property of toxic aminoglycosides and was not seen with non-toxic fragments or derivatives of these drugs (Schacht, 1976).

The formation of the aminoglycoside-polyphosphoinositide complex should have strong influences on the integrity of the plasma membrane. The polyphosphoinositides are believed to control membrane-bound calcium (Kai and Hawthorne, 1969) which serves important functions in the stability and excitability of membranes. When neomycin binds to the polyphosphoinositides, calcium is displaced, as we have shown previously (Lodhi et al., 1976). In addition, we have provided evidence that the formation of a polyphosphoinositide-neomycin complex leads to disturbances of the structure of lipid membranes (Schacht et al., 1977) resulting in an increased permeability of the cell (Stockhorst and Schacht, 1977). Such a disturbance of membrane structure and function may be sufficient to explain most of the actions of the drugs. It seems attractive, however, to speculate that this membrane action is the first and necessary prerequisite for the drug to enter the cell. Inside the cell, the drug then may exert further toxic actions at different locations (Tachibana et al., 1976; Jarlstedt and Bagger-Sjöbäck, 1977).

It is interesting to note in this context that the polyphosphoinositides are not uniformly distributed among body tissues. Besides brain, which has a high polyphosphoinositide content, but is protected from the aminoglycosides by the blood brain barrier, the only organ that shows a high content of these lipids is the kidney (Hauser and Eichberg, 1973). This correlates well with clinical observations of the pathology following drug intoxication. The amounts of these lipids in the inner ear are presently being measured and our labeling studies with ^{32}P already indicate that the polyphosphoinositides are highly active constituents of cochlear tissues. It therefore seems appropriate to assume that the presence of these lipids makes a tissue susceptible to the attack by aminoglycoside antibiotics. Consequently, we should attempt to prevent the interactions of neomycin with the polyphosphoinositides. If the aminoglycosides could be modified accordingly, we might obtain a drug which no longer is toxic to the inner ear or the kidney. It would remain to be established whether such a drug can still be antibacterially active. This appears possible since the site of action in bacteria is at a ribosomal protein. Polyphosphoinositides do not occur in bacteria.

The experiments presented here provide a novel approach to the question of the molecular mechanism of aminoglycoside toxicity. The isolation of polyphosphoinositides from cochlear and renal tissues by affinity chromatography on neomycin is strong evidence for an important role of these lipids in aminoglycoside toxicity.

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