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ACTION OF NEOMYCIN ON THE METABOLISM OF POLYPHOSPHOINOSITIDES IN THE GUINEA PIG KIDNEY*

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Abstract—The effect of neomycin on the metabolism of phospholipids was investigated in the guinea pig kidney both *in vivo* and *in vitro*. *In vivo*, after an intraperitoneal injection of [^{32}P]orthophosphate, the polyphosphoinositides (phosphatidylinositol phosphate and phosphatidylinositol diphosphate) were the most rapidly labeled phospholipids reaching maximal radioactivity within 40 min. The labeling of these lipids paralleled that of the nucleotide phosphate pool. After chronic treatment of animals with neomycin (100 mg/kg body weight daily for 6 days), phosphate incorporation into the polyphosphoinositides was decreased whereas incorporation into other lipids remained unaffected. Also, the amount of phosphatidylinositol diphosphate was reduced from 270 to 170 nmoles/g of kidney. No significant changes in phospholipid labeling were found in the liver. Experiments *in vitro* with kidney homogenates demonstrated inhibition of labeling of phosphatidylinositol diphosphate from [γ-^{32}]ATP and inhibition of the enzymatic hydrolysis of previously labeled [^{32}P]phosphatidylinositol phosphate and diphosphate in the presence of neomycin. A direct antibiotic–lipid interaction is discussed as a mechanism for the observed effects of neomycin on renal polyphosphoinositide metabolism.

The chronic toxicity of aminoglycosidic antibiotics (neomycin, streptomycin and related compounds) to the kidney and the inner ear has long been recognized as a problem in chemotherapy [1]. Both tissues accumulate these antibiotics above serum drug levels [2, 3], and eventually kidney failure and deafness may result. In contrast to the well-established clinical and morphological observations [1, 4–6], the biochemical mechanisms underlying these tissue specific toxic actions remain unclear.

We have previously demonstrated an effect of neomycin on polyphosphoinositide labeling in inner ear tissues *in vivo* [7, 8]. Polyphosphoinositides are metabolically highly active constituents of nervous and secretory tissues, and have been suggested to be involved in events related to changes in membrane permeability and ion transport [9, 10]. Of non-nervous tissues, kidney has a relatively high content of polyphosphoinositides [11, 12], and a rapid turnover of the monoesterified phosphate groups of these lipids has been reported for rat kidney [12]. Anatomical, physiological, and immunological similarities between inner ear tissues and the kidney [13] have been demonstrated, and some congenital syndromes exhibit both renal and hearing defects [14, 15]. In addition, a number of drugs, notably diuretics and aminoglycosides, exert pharmacological actions on the kidney as well as the inner ear. These considerations led us to investigate the influence of neomycin on phospholipid metabolism in the kidney.

MATERIALS AND METHODS

Labeling and separation of tissue lipids. Male albino guinea pigs (BioLabs, St. Paul, MN) weighing 300–400 g were injected intraperitoneally with carrier-free sodium [^{32}P]orthophosphate (1 mCi/kg body wt) in sterile saline and killed by decapitation. Tissues were quickly removed, weighed and homogenized in 10 vol. of ice-cold 10% (w/v) trichloroacetic acid with a Polytron homogenizer (Brinkman Instruments, Westbury, NY).

Total lipid extracts were obtained from the trichloroacetic acid homogenates with the biphasic system of chloroform–methanol–HCl, according to the method described by Hajra *et al.* [16]. The lipid extracts were resolved by thin-layer chromatography on Silica gel (Brinkman, Silica gel 60) using two different solvent systems: solvent 1, chloroform–methanol–conc. aqueous ammonia–water (45:45:3.5:11, v/v) and solvent 2, chloroform–methanol–glacial acetic acid–water (50:32:11:3, v/v). Lipids were identified by co-chromatography with commercial standards (Supelco, Bellefonte, PA) and polyphosphoinositides prepared from ox brain according to Hendrickson and Ballou [17]. [^{32}P]lipids on thin-layer chromatography plates were located by autoradiography on Kodak No-Screen X-ray films (Eastman Kodak, Rochester, NY), scraped into vials and counted by liquid scintillation spectrometry.

For the analysis of polyphosphoinositide content of kidneys, lipids were extracted and purified as described by Hauser and Eichberg [11].

Experiments in vitro. Guinea pig kidneys were homogenized in 9 vol. of 0.25 M sucrose using a glass–glass homogenizer and centrifuged at 1000 *g* for 20 min. The pellet was discarded and the resulting supernatant fraction (henceforth referred to as "homogenate") was frozen-and-thawed four times to disrupt mitochondria. For labeling of phospholipids by [γ-^{32}P]ATP, incubations were performed at 37° in air in a shaking water bath. Homogenates (0.43 mg protein/incubation) were preincubated in 50 mM

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sodium HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, for 15 min in the presence or absence of neomycin (in 0.25 ml). Incubations were continued after the addition of 15 mM $MgCl_2$ and 3 mM Tris [γ-^{32}P]ATP (final concentrations in 0.5 ml final volume). The reactions were terminated by the addition of 1.5 ml of chloroform–methanol (1:2, v/v). Lipids were extracted after the addition of 0.5 ml of 2.4 N HCl and 1.0 ml chloroform. The resulting upper layer was re-extracted with 1.0 ml chloroform. The combined lower phases were then washed with 2.0 ml methanol–0.6 N HCl (1:1, v/v). Lipid separation and determination of radioactivity were performed as described above.

Hydrolysis of polyphosphoinositides was studied using ^{32}P-labeled lipids extracted from guinea pig kidneys after intraperitoneal injection of $^{32}P_i$. The total lipid extract was neutralized, dried under N_2 and suspended in 50 mM sodium HEPES, pH 7.4, by sonication. After preincubation of the homogenate (0.35 to 0.60 mg protein) in 50 mM sodium HEPES, pH 7.4, 2 mM $MgCl_2$ and 2 mM KF in 0.25 ml for 15 min in the presence or absence of neomycin, aliquots (0.25 ml) of the ^{32}P-labeled lipid suspensions were added and incubations continued. Hydrolysis was stopped by the addition of 1.5 ml of chloroform–methanol (1:2, v/v).

Other analytical procedures. Nucleotide phosphate in the trichloroacetic acid supernatant fractions from tissue homogenates was absorbed on charcoal, washed and hydrolyzed for 10 min in 1 N HCl [18], and released inorganic phosphorus was assayed spectrophotometrically [19]. Lipid phosphorus was determined after ashing [20]. [γ-^{32}P]ATP radioactivity in the HCl-soluble upper layers from incubations *in vitro* was measured by the method of Huang [21]. Protein was determined by the method of Lowry *et al.* [22].

For neomycin determination, five animals were injected subcutaneously with neomycin (100 mg/kg body wt) daily for 6 days and killed 24 hr after the last injection. Kidneys were homogenized with a Polytron in 20 vol. of 0.1 M Na-phosphate, pH 8.0, and aliquots were assayed microbiologically [23–25]. Blanks and neomycin standards were determined in the presence of control tissue.

Materials. Carrier-free [^{32}P]orthophosphate was obtained from New England Nuclear (Boston, MA), and Sephadex G-25-300 and HEPES from Sigma Chemical Co. (St. Louis, MO). Neomycin B sulfate was a gift from the Upjohn Co. (Kalamazoo, MI). [γ-^{32}P]ATP was prepared enzymatically [26] and purified by ion-exchange chromatography [27].

RESULTS

Labeling of phospholipids in vivo. The time course of ^{32}P-incorporation into the phospholipids of the guinea pig kidney (Fig. 1) shows that phosphatidylinositol phosphate (PhIP) and diphosphate ($PhIP_2$) were rapidly labeled reaching a maximum 20–40 min after injection of $^{32}P_i$, followed by loss of label with increasing time. The time course of labeling of the polyphosphoinositides followed closely the appearance of radioactivity in the acid-labile nucleotide phosphate pool. These results agree well with the observations of Tou *et al.* [12] for the rat kidney.

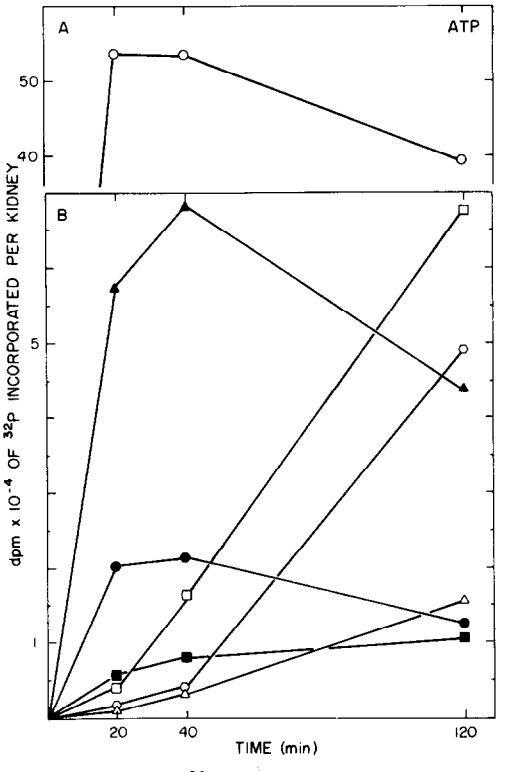

Fig. 1. Time course of ^{32}P-incorporation into (A) ATP and (B) phospholipids in the guinea pig kidney *in vivo*. Animals were injected intraperitoneally with 1 mCi $^{32}P_i$/kg body wt. Kidneys were analyzed as described in Materials and Methods. Each time point represents the average value from four kidneys. Key: ATP (○); $PhIP_2$ (▲); PhIP (●); PhA (■); PhI (△); PhE (□); and PhC (◯).

Phosphatidic acid (PhA)* also revealed a rapid pattern of labeling and reached a plateau after 40 min. The initial labeling of the other phospholipids proceeded much more slowly.

Effects of neomycin treatment. Guinea pigs received subcutaneous injections of neomycin sulfate (100 mg neomycin/kg body wt) daily for 6 or 7 days. At this time, the drug had accumulated in the kidney to about millimolar concentration and kidney weight had increased significantly (Table 1). The amounts of protein, total phospholipid and soluble phosphates were also elevated but when these values were normalized to tissue weight, no significant changes were seen in these compounds between drug-treated and control animals. There were also no changes in phosphate incorporation into the quantitatively major phospholipids (Table 2) when incorporation was calculated from the specific radioactivity of the precursor, [γ-^{32}P]ATP. However, incorporation into $PhIP_2$ and to a lesser extent into PhIP was reduced. This difference between the polyphosphoinositides and the other phospholipids was confirmed in a total of five experiments with drug treatment varying from 6 to 10 days. Concomitant with the reduced incorporation, the

* Abbreviations used are: PhA = phosphatidic acid (1,2-diacyl-sn-glycero-3-phosphate); PhC, PhE, PhS = phosphatidylcholine, -ethanolamine, -serine; and PhI, PhIP, $PhIP_2$ = phosphatidylinositol, phosphate, diphosphate.

Table 1. Effects of neomycin treatment*

	Control animals	Drug-treated animals
Body wt (g)	350 ± 11	337 ± 5
Kidney wt (g)	1.3 ± 0.1	2.0 ± 0.1†
Liver wt (g)	11.9 ± 0.6	11.8 ± 0.9
Kidney analysis (per g wet wt)		
Protein (mg)	156 ± 12	136 ± 13‡
P_i (μmoles)	6.2 ± 0.4	5.6 ± 0.8
Nucleotide ~ P (μmoles)	1.7 ± 0.1	1.7 ± 0.1
Lipid-P (μmoles)	21.0 ± 1.5	19.3 ± 1.8
Neomycin (μmoles)		0.94 ± 0.14§
Liver analysis (per g wet wt)		
Protein (mg)	159 ± 9	163 ± 11
P_i (μmoles)	6.7 ± 0.6	6.6 ± 1.2
Nucleotide ~ P (μmoles)	1.4 ± 0.1	1.3 ± 0.1
Lipid-P (μmoles)	17.5 ± 2.2	17.6 ± 2.6

* Guinea pigs received subcutaneous injections of neomycin (100 mg/kg body wt) daily for 7 days and were sacrificed on day 8. Six animals were analyzed/group as described in Methods. Values are expressed as means ± S. D.

† $P < 0.001$.

‡ $0.05 < P < 0.10$.

§ From a parallel experiment; see Methods.

amount of $PhIP_2$ was lowered after 6 days of neomycin injections from 270 ± 60 nmoles/g of tissue (five control animals) to 170 ± 60 nmoles/g of tissue (five treated animals; $0.05 > P > 0.02$).

In liver, no drug-induced changes were observed in weight, content of protein, phospholipid or soluble phosphates (Table 1). After intraperitoneal injection of $^{32}P_i$, the most highly labeled phospholipid was phosphatidylcholine (Table 2). The absence of a large incorporation into the polyphosphoinositides may reflect the lower content of these lipids [28] or may indicate a different metabolic role in this tissue. After drug treatment, there were no significant differences in the P_i incorporation into any of the phospholipids including PhIP and $PhIP_2$.

Studies in vitro. In order to gain some understanding of the possible mode of action of neomycin on the metabolism of polyphosphoinositides, experiments *in vitro* were designed to investigate the effect of the antibiotic on the synthesis and breakdown of these phospholipids.

Lipids were labeled *in vitro* by incubation of kidney homogenates (1000 *g* supernatant) with [γ-^{32}P]ATP. Under these conditions, the label appeared mainly in PhIP, $PhIP_2$ and PhA. Calculations of the rate of synthesis of $PhIP_2$ and PhIP from the initial slopes (Fig. 2) and the specific radioactivity of [γ-^{32}P]ATP yield values of 15 and 23 pmoles phosphate incorporated/min/mg of protein, respectively, under the conditions of the assay. These values are in good agreement with the observed activities of PhIP kinase [29] and PhI kinase [30] in homogenates of rat kidney cortex.

The time course of phospholipid labeling from [γ-^{32}P]ATP showed an inhibition of ^{32}P-incorpor-

Table 2. Effect of neomycin on phosphate incorporation into phospholipids *in vivo**

	Control animals	Drug-treated animals
Kidney (nmoles P_i incorporated/g tissue)		
Phosphatidylethanolamine	108 ± 18	92 ± 11
Phosphatidylcholine	42 ± 8	41 ± 11
Phosphatidic acid	31 ± 3	39 ± 10
Phosphatidylinositol (+ serine)	31 ± 5	31 ± 9
Phosphatidylinositol phosphate	99 ± 14	73 ± 7†
Phosphatidylinositol diphosphate	317 ± 50	199 ± 23‡
Liver (nmoles P_i incorporated/g tissue)		
Phosphatidylethanolamine	76 ± 4	80 ± 8
Phosphatidylcholine	120 ± 19	116 ± 12
Phosphatidylinositol (+ serine)	104 ± 19	94 ± 9
Phosphatidylinositol phosphate	43 ± 2	39 ± 3
Phosphatidylinositol diphosphate	63 ± 5	58 ± 9

* Guinea pigs received subcutaneous injections of neomycin (100 mg/kg body wt) daily for 6 days. Controls received saline. On day 7 animals were given 1 mCi $^{32}P_i$/kg body wt intraperitoneally. Six tissues were analyzed/group as described in Methods. Values are expressed as means ± S. D.

† $0.02 < P < 0.05$.

‡ $0.01 < P < 0.02$.

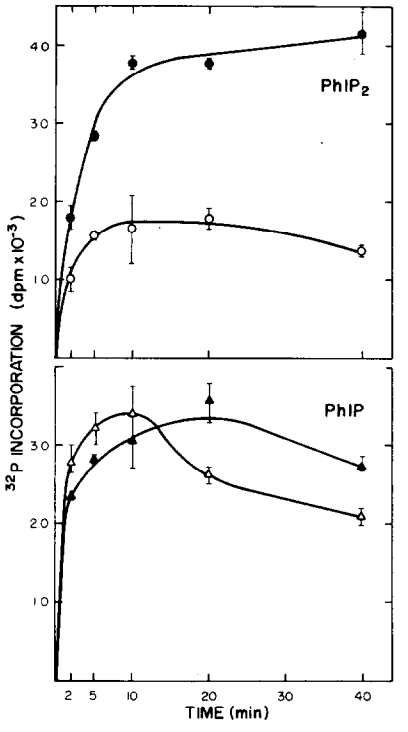

Fig. 2. Time course of labeling of phospholipids *in vitro* from [γ-^{32}P]ATP. Kidney homogenates (0.43 mg protein) were incubated as described in Methods in the presence (open symbols) or absence (closed symbols) of 10^{-4} M neomycin. [γ-^{32}P]ATP was 3 mM (73.3×10^6 dis./min/μmole).

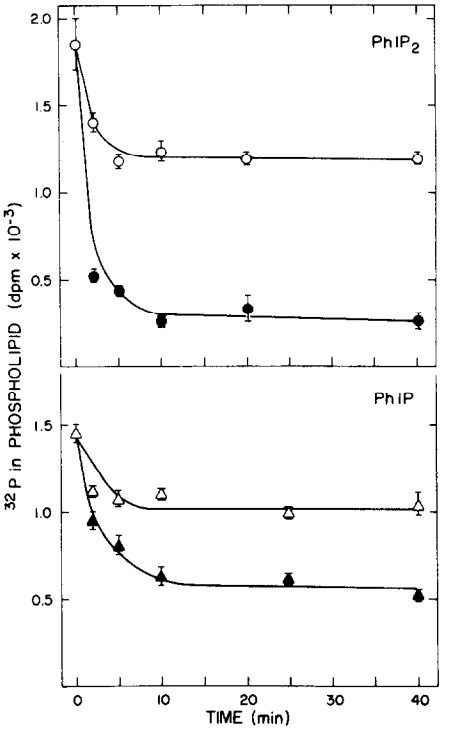

Fig. 4. Time course of enzymatic hydrolysis of previously labeled [^{32}P]lipids. Desalted kidney homogenates were incubated in the presence (open symbols) or absence (closed symbols) of 10^{-4} M neomycin as described in Methods.

ation into $PhIP_2$ in the presence of neomycin (Fig. 2). This inhibition of labeling of $PhIP_2$ was dependent on the concentration of antibiotic ($I_{50} = 10^{-5}$–10^{-4} M, Fig. 3). An increase in the labeling of PhIP was observed only at 10^{-2} M neomycin. Measurements of [γ-^{32}P]ATP at the end of each incubation showed that the rate of ATP hydrolysis was not changed significantly (less than 10 per cent) in the presence of neomycin.

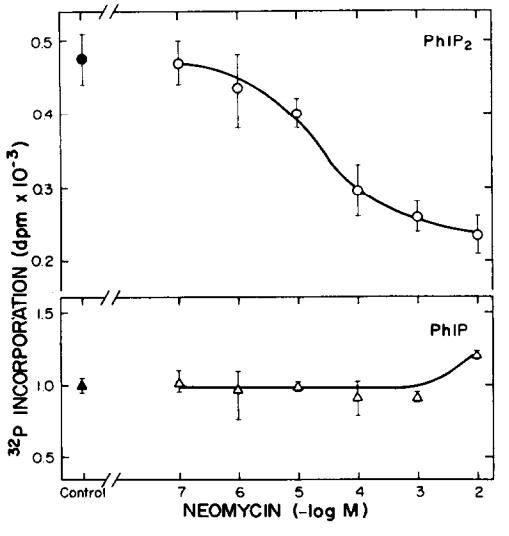

Fig. 3. Effect of varying concentrations of neomycin on the labeling of phospholipids *in vitro*. The incubation system was the same as that described in Fig. 2, except that the incubation time was 10 min and the neomycin concentration was varied as shown. Closed symbols indicate incubations in the absence of neomycin.

Hydrolysis of phospholipids was measured using ^{32}P-labeled total lipid extracts obtained from guinea pig kidneys after intraperitoneal injections of $^{32}P_i$. In order to remove inorganic phosphate, which has been shown to inhibit $PhIP_2$ phosphomonoesterase activity in rat kidney preparations [31], the kidney homogenate was desalted by passage through Sephadex G-25 equilibrated with 0.25 M sucrose. Incubation of this desalted preparation with sonicated suspensions of phospholipids previously labeled with $^{32}P_i$ showed that PhIP and $PhIP_2$ were rapidly hydrolyzed. This enzymatic hydrolysis was blocked by the antibiotic (Fig. 4) in a dose-dependent fashion (Fig. 5, $I_{50} = 10^{-5}$–10^{-4} M).

DISCUSSION

Few reports on the phospholipids of the kidney have been concerned with polyphosphoinositides. In this study, we have demonstrated a rapid labeling of PhIP and $PhIP_2$ in the guinea pig kidney *in vivo*. Labeling of the polyphosphoinositides has been shown to proceed by phosphorylation of phosphatidylinositol (PhI) by [γ-^{32}P]ATP according to the following reactions [29, 32]:

$$PhI + [\gamma\text{-}^{32}P]ATP \rightarrow [^{32}P]PhIP + ADP \quad (1)$$

$$PhIP + [\gamma\text{-}^{32}P]ATP \rightarrow [^{32}P]PhIP_2 + ADP \quad (2)$$

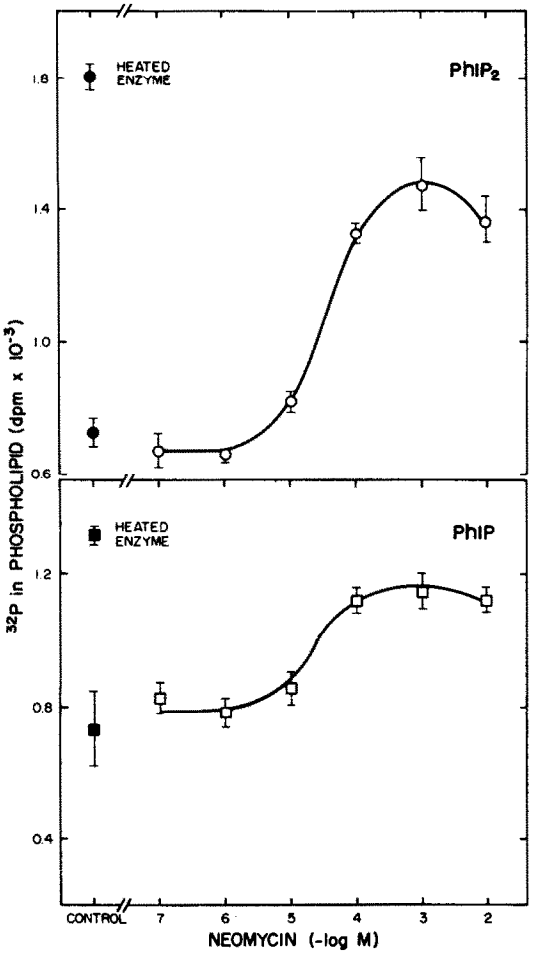

Fig. 5. Effect of varying concentrations of neomycin on the enzymatic hydrolysis of previously labeled [^{32}P]lipids. Conditions were the same as indicated in Fig. 4. Incubations were performed for 5 min at varying neomycin concentrations. Control incubations were carried out in the absence of neomycin and with an inactivated homogenate ("heated enzyme," 15 min at 90°) respectively. The latter also indicates the amount of [^{32}P]lipid present at the start of the incubation.

The rapid equilibration of ^{32}P in the polyphosphoinositides with the acid-labile nucleotide pool (the immediate precursor) and the lag observed in the labeling of PhI indicate that labeling is confined to the monester phosphate groups according to reactions 1 and 2, and is not introduced by *de novo* synthesis via phosphatidylinositol. This has also been observed in the pig [33] and rat kidney [12].

This metabolism is apparently affected by neomycin *in vivo* and *in vitro*. *In vivo*, neomycin accumulates in the kidney to a millimolar concentration after 6 days of drug injection. Similar findings have been reported for streptomycin [2] and kanamycin [3]. Early morphological and physiological damage inflicted by aminoglycosides includes tubular necrosis, polyuria, albuminuria and decreased urea clearance and should be expected from the dosage of neomycin given in this experiment [1, 5, 6, 34]. The effect of neomycin on the polyphosphoinositides is evident in the labeling studies where the rate of P_i-incorporation is decreased considerably, whereas no such effect is seen on the quantitatively major phospholipids. In agreement with this, the measured total phospholipid content is unchanged but the content of phosphatidylinositol diphosphate is significantly lower after drug treatment. Since $PhIP_2$ represents only about 1 per cent of the total renal phospholipids, this decrease is not reflected in the total lipid determination. Previous biochemical studies which reported renal phospholipid metabolism to be unchanged under the influence of dihydrostreptomycin did not investigate the polyphosphoinositides [35, 36].

The decreased labeling of polyphosphoinositides after neomycin intoxication was observed in the kidney, but not in the liver. This agrees well with clinical and pathological findings that the kidney and the inner ear are the primary organs affected by aminoglycosidic antibiotics after systemic injections [1]. Similar decreases in the labeling of polyphosphoinositides by $^{32}P_i$ were found in inner ear tissues after ototoxic damage by neomycin [6, 7]. Although brain has the highest content of PhIP and $PhIP_2$ of all body tissue, it is protected from the action of aminoglycosides by the blood–brain barrier [2].

While the experiments *in vivo* point to a possible correlation between the biochemical and pharmacological actions of the drug, they leave open the question of the possible biochemical mechanism. Inhibition of kinase reactions or stimulation of hydrolysis will lead to a decreased labeling of polyphosphoinositides and decreased content of $PhIP_2$ as observed in our studies *in vivo*. The latter possibility is not supported by the results *in vitro* as the hydrolysis of both PhIP and $PhIP_2$ is blocked by neomycin. In support of the first alternative, labeling of $PhIP_2$ via the kinase reaction is strongly inhibited by neomycin but labeling of PhIP is largely unaffected. The levels of neomycin showing these effects *in vitro* are well below the drug concentration reached in the kidney after chronic drug treatment.

A mechanism which would account for the observations, both *in vivo* and *in vitro*, is suggested by the fact that polyphosphoinositides can form complexes with polycations [37], including neomycin [38]. We have recently demonstrated that changes by neomycin of polyphosphoinositide labeling in guinea pig brain subcellular fractions can be explained by such a direct drug–lipid interaction [39].

Binding of the aminoglycoside to the acidic polyphosphoinositides would effectively lower the availability of these lipids for both kinase and hydrolase actions. *In vitro*, labeling of $PhIP_2$ (reaction 2) depends on the availability of PhIP, which would be lower in the presence of neomycin, and ^{32}P-incorporation into $PhIP_2$ would be diminished. Labeling of PhIP would not be expected to be significantly lowered by neomycin since the PhI content would not be limiting. On the contrary, an increase in [^{32}P]PhIP would be expected in the presence of neomycin due to the inhibition of its hydrolysis and of labeling of $PhIP_2$ (reaction 2), and this is observed at the higher drug concentrations. Similarly, binding of neomycin to $PhIP_2$ and PhIP would decrease the hydrolysis of these lipids.

Inhibition of phosphomonoesterase reactions by substrate complexing by neomycin can also explain the decreased labeling of polyphosphoinositides *in*

vivo. If steady state levels of PhIP and $PhIP_2$ are maintained by the combined action of kinases and phosphomonoesterases, reduction of hydrolytic activity due to lowered substrate availability would result in a slower turnover of $PhIP_2$ and PhIP and in a decrease in labeling of these two lipids. Chronically, an inhibition of PhIP conversion to $PhIP_2$ should lead to lowered concentrations of $PhIP_2$ which we indeed observed. Thus, the assumption of a direct antibiotic–polyphosphoinositide interaction is sufficient to explain the experimental observations. Nevertheless, a neomycin effect directly on some of the enzymes involved in the turnover of these phospholipids cannot be ruled out at this point.

Polyphosphoinositides are believed to be important lipid constituents of nervous and secretory tissues [9, 40]. The kidney has a large complement of plasma membranes and contains appreciable quantities of PhIP and $PhIP_2$ [12]. These phospholipids have a high affinity for Ca^{2+} and Mg^{2+} ions, and it has been postulated that they may play an important role in the active transport of cations and regulation of membrane permeability to ions. It has been speculated [12] that these lipids participate in some important function in the tubule membranes during secretion and reabsorption of solutes from the lumen of the tubules. Complexing of the polyphosphoinositides by the polycationic antibiotic would be expected to interfere with the functional role of these lipids in the kidney. In addition, changes in membrane integrity induced by such binding could facilitate the penetration of these drugs into the cell where they may exert further toxic effects [3].

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