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Neomycin Inhibition of Adenosine Triphosphatase: Evidence for a Neomycin-Phospholipid Interaction

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The inhibition of canine renal sodium potassium adenosine triphosphatase (ATPase) by neomycin was examined. Neomycin inhibited ATPase nearly maximally at 0.02 mM. The inhibition was temperature dependent with a decrease in inhibition occurring at temperatures below 21°C, a temperature which corresponded to a change in activation energy of the ATPase as determined by Arrhenius plot. Preincubation of the ATPase with phosphoinositides was found to prevent the inhibition by neomycin. Other phospholipids were not found to prevent the inhibition. These results indicate a possible interaction between neomycin and the phosphoinositides of the ATPase complex.

Two significant toxicities associated with aminoglycoside antibiotics are nephrotoxicity and ototoxicity. Schacht and co-workers have shown that aminoglycosides alter the metabolism of phosphatidylinositol in the cochlea and have hypothesized that there is an interaction between aminoglycosides and phosphatidylinositol as an explanation of the mechanism of the ototoxicity of this class of drugs (5). Darrow has demonstrated an inhibition of sodium potassium adenosine triphophatase (ATPase) by aminoglycosides (W. R. Darrow, Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio, 1969). Since phospholipids are important for the function of sodium potassium ATPase (2), we have examined the inhibition of renal sodium potassium ATPase by the aminoglycoside, neomycin, to determine whether the inhibition is consistent with a phospholipid interaction with the aminoglycoside. It is possible that such studies will lead to an increased understanding of the nephrotoxicity and otoxicity of aminoglycoside antibiotics.

MATERIALS AND METHODS

Canine renal sodium potassium ATPase (EC 3.6.1.3.; grade IV), phosphoenolpyruvate, lactic dehydrogenase, pyruvate kinase, the reduced form of nicotinamide adenine dinucleotide (NADH), neomycin sulfate, phosphatidylcholine, phosphatidylserine, phosphatidylinositol monophosphate, phosphatidylinositol diphosphate, phosphatidylethanolamine, and cardiolipin were purchased from Sigma Chemical Co., St. Louis, Mo. Sodium potassium ATPase activity was determined spectrophotometrically by a modification of the method of Pullman et al. (7) by measuring the enzymatically coupled oxidation of NADH. Reaction mixtures contained in a volume of 1 ml: 100 mM NaCl, 10 mM KCl, 5 mM $NgCl_2$, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 5 mM adenosine 5'-triphosphate (ATP), 1.5 mM NADH, 1.5 mM phosphoenolpyruvate, 2 U of lactic dehydrogenase per ml, 20 U of pyruvate kinase per ml, and 0.1 U of sodium potassium ATPase per ml. Reactions were initiated by the addition of 200 μl of an ATPase solution which contained 0.25 M sucrose, 20 mM L-histidine, 1 mM ethylenediaminetetraacetic acid and 0.1% (wt/vol) deoxycholic acid. Neomycin was dissolved in 50 mM $NaPO_4$ buffer (pH 7.4). In the phospholipid addition experiments phospholipids were added in a volume of 0.25 ml in diluents as follows. For phosphatidylinositol, phosphatidylinositol monophosphate, and phosphatidylinositol diphosphate we used water; for phosphatidylcholine and cardiolipin we used ethanol-water, 1:75; for phosphatidylserine, chloroform-methanol, 1:200, was used; for a phosphatidylethanolamine, chloroform was used in combination (1:100) with reaction mixture.

RESULTS

The inhibition of sodium potassium ATPase by neomycin is shown in Fig. 1. The dose-response curve demonstrates that at concentrations of 0.02 mM neomycin or greater there was more than 90% inhibition of the ATPase. In these experiments it was necessary to preincubate the ATPase with the neomycin before the addition of ATP for inhibition to occur. A preincubation time of 15 min at 37°C was based on the observations that preincubation times of 5 and 10 min before the addition of ATP resulted, respectively, in 85 and 100% of maximal inhibition. Control ATPase preparations did not decrease in activity during the preincubation period. To determine whether neomycin directly affected the enzyme-coupled system used to measure ATPase activity, neomycin at a concentration of 0.5 mM was added to the coupling system in the presence of 0.01 mM adenosine 5'-diphosphate. This high concentration of neo-

mycin did not produce any effect upon the rate of oxidation of NADH. Figure 1 also demonstrates that the extent of inhibition of ATPase by neomycin was correlated with the optical density of the solution containing the ATPase and neomycin. The optical density of the solution increased as the extent of inhibition increased (Fig. 1). The optical density measurements were made after the 15-min preincubation. An examination of optical density changes during preincubation revealed that 100% of the final optical density value occurred by 10 min of preincubation.

The extent of inhibition was also found to be dependent on the temperature of preincubation (Fig. 2). A concentration of 0.55 mM neomycin was used. At lower preincubation temperatures, neomycin was less able to inhibit ATPase. Nearly maximal inhibition was not seen until the preincubation temperature was above 20°C. The significance of the temperature needed for maximal inhibition was investigated by an examination of the activation energy of the ATPase preparation. The activity of the ATPase was measured as a function of the temperature of the reaction mixture. An Arrhenius plot of the data obtained (Fig. 3) demonstrates that there was a change in the slope of the plotted data at approximately 21°C, thus indicating a change in activation energy of the ATPase at that point.

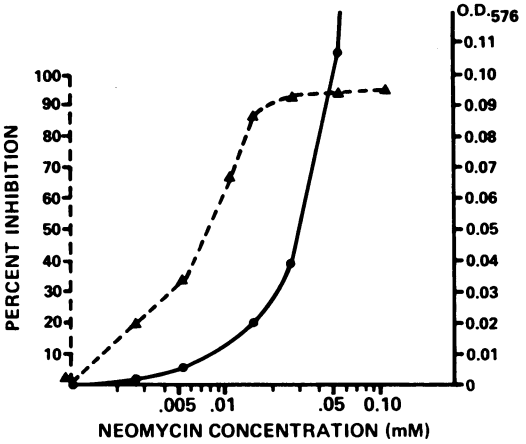

FIG. 1. *Dose response of inhibition of ATPase by neomycin and optical density of the solution.* (▲) *Percent inhibition;* (●) *optical density of the solution.*

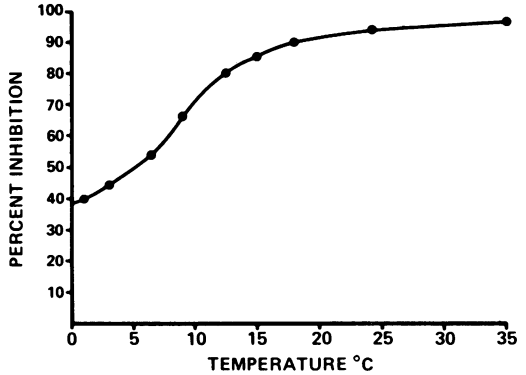

FIG. 2. *Temperature dependence of the ability of neomycin to inhibit ATPase.*

The effect of phospholipids known to be in the ATPase complex upon the ability of neomycin to inhibit ATPase was examined since it is possible that neomycin may interact with these phospholipids (Table 1). In these experiments the phospholipid was added with the neomycin. Phosphatidylinositol and its mono- and diphosphates were able to prevent the inhibition of ATPase by neomycin (Table 1). Addition of phosphatidylinositols after the preincubation of ATPase with neomycin failed to reverse the inhibition. The other phospholipids were unable to prevent the inhibition, even though they were inherently able to enhance ATPase activity. Phosphatidylinositol and its mono- and diphosphates were similar in their ability to prevent the inhibition of ATPase by neomycin as demonstrated by dose-response curves (Fig. 4). To determine whether the phospholipids affected the enzyme-coupled system used to measure ATPase activity, each phospholipid at a concentration of 0.02 mM in the presence of its diluent was added to the enzyme-coupling system in the presence of 0.01 mM adenosine 5'-diphosphate. Under these conditions none of the phospholipids altered the rate of oxidation of NADH.

DISCUSSION

An interpretation of the results presented in this paper is that neomycin may interact with

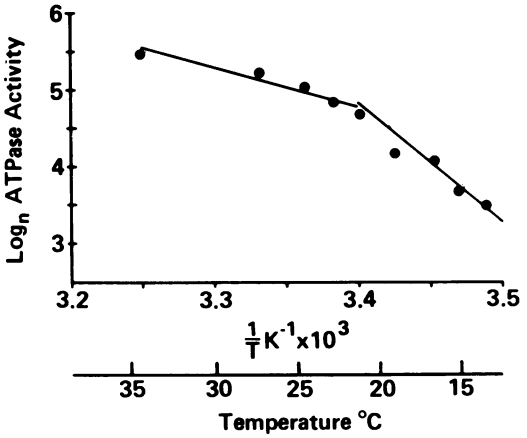

FIG. 3. *Arrhenius plot of ATPase activity. Upper scale on the abscissa is the reciprocal of the temperature in °K.*

TABLE 1. *Effect of phospholipids on ability of neomycin to inhibit ATPase*

Phospholipid	% ATPase activity (mean ± SD)[a] after treatment:		
	Lipid (0.02mM)	Neomycin (0.04mM)	Neomycin (0.04 MM) + lipid (0.02mM)
Phosphatidylinositol	126 ± 3	40 ± 5	133 ± 5
Phosphatidylinositol monophosphate	138 ± 16	39 ± 11	138 ± 5
Phosphatidylinositol diphosphate	104 ± 1	49 ± 1	157 ± 1
Phosphatidylcholine	118 ± 3	51 ± 8	63 ± 8
Phosphatidylserine	157 ± 26	43 ± 16	42 ± 14
Phosphatidylethanolamine	117 ± 5	9 ± 2	22 ± 6
Cardiolipin	74 ± 2	38 ± 2	32 ± 2

[a] 100% = ATPase activity in presence of the diluent of the phospholipid. SD, Standard deviation.

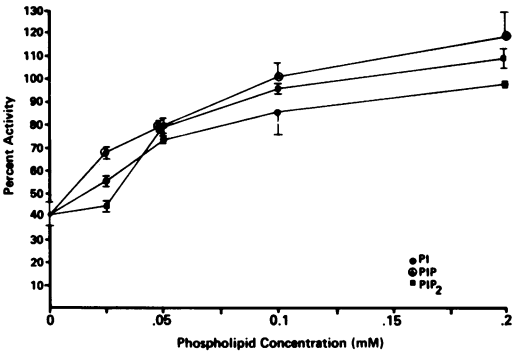

FIG. 4. *Dose response of the prevention of neomycin inhibition of ATPase by phosphoinositides. Points are the mean ± standard deviation. PI, Phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol diphosphate.*

phospholipids in the ATPase complex. Sodium potassium ATPase is believed to be a complex composed of a pair of each of two subunit proteins surrounded by a phospholipid annulus (9). The fluidity of the phospholipid annulus affects the activity of the ATPase and may alter the activation energy of the ATPase. A change in activation energy is indicated by a break in the Arrhenius plot of sodium potassium ATPase. Therefore an interpretation of the break at 20°C that we observed and which is consistent with Arrhenius plots obtained by others (1, 6) is that of a phase transition in the lipid annulus (4). Above 20°C the lipids may be in a relatively fluid state as compared to below 20°C. The decreased ability of neomycin to inhibit ATPase below 20°C (Fig. 2) may indicate that the fluidity of the lipids may be important to permit neomycin to interact with ATPase. Another explanation for the neomycin temperature sensitivity is that neomycin may exert its inhibition by causing a decrease in lipid fluidity. Therefore, below 20°C lipid fluidity would already be decreased and thus neomycin would show less inhibitory effect. A similar hypothesis has been proposed by Gruener and Avi-Dor (3) as an explanation for an observed decreased sensitivity of ATPase to ouabain at temperatures below the break point of the Arrhenius plot. A further indication that neomycin may form a complex with lipids of the ATPase is the increase in optical density that we observed upon incubation of the ATPase with increasing concentrations of neomycin (Fig. 1). Although many explanations may be possible for this change, Stockhorst and Schacht have observed neomycin-polyphosphoinositide interactions which have resulted in similar optical density changes (8).

More compelling evidence for a lipid interaction comes from our data on the ability of phospholipids to prevent inhibition by neomycin. The prevention of inhibition appears to be specific with phosphatidylinositol and its mono- and diphosphate analogs. These lipids are negatively charged, and it is possible that the positively charged neomycin combines with the negatively charged lipids. It is reasonable that there may be specificity to the lipid interactions since cardiolipin, a negatively charged phospholipid, did not prevent the inhibition of ATPase by neomycin. Furthermore, the prevention of inhibition is not related to a stimulating effect of the added phsopholipid. Although phosphatidylserine was able to cause a greater than 50% increase in base-line ATPase activity (Table 1), it was unable to prevent inhibition.

In conclusion, the results we obtained suggest an interaction between neomycin and the phospholipids of the ATPase complex. We have obtained evidence suggesting that neomycin may interact specifically with the phosphatidylinositols. One may speculate that the mechanism of inhibition of ATPase may be related to an alteration in lipid fluidity caused by the neomycin. The inhibition of ATPase by neomycin may disrupt cellular function and thus be related to the toxicity of aminoglycosides.

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