Inhibition of neural phospholipase D activity by aminoglycoside antibiotics

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The effects of aminoglycoside antibiotics on phospholipase D (PLD) activity were investigated in permeabilized NG108-15 cells and in isolated rat brain membranes. Neomycin inhibited guanosine 5'-[γ -thio]triphosphate-stimulated PLD activity in digitonin-permeabilized NG108-15 cells in a concentration-dependent manner (50% inhibition at 100 μ M). Neomycin similarly inhibited PLD activity present in rat brain membranes and assayed *in vitro* with [3 H]phosphatidylcholine as substrate (50% inhibition at 65 μ M). Other aminoglycosides tested (kanamycin, geneticin and streptomycin) were nearly equipotent inhibitors of rat brain PLD. These results indicate that aminoglycoside antibiotics inhibit phosphatidylcholine-PLD activity with comparable and sometimes greater potency than their well known inhibition of phosphoinositide-phospholipase C. The possibility that PLD inhibition could mediate some of the toxic side effects of aminoglycosides is suggested.

INTRODUCTION

Extracellular signals (neurotransmitters, hormones, growth factors etc.) affect cell function by interacting with specific receptors located on the cell surface. Evidence gathered over the last few years indicates that the signal-dependent activation of phospholipase D (PLD) may constitute a novel pathway for transducing such signals across the plasma membrane. PLD-catalysed hydrolysis of phosphatidylcholine (PC) is rapidly activated, in the same cell types, by many of the Ca^{2+} -mobilizing agonists that are known to engage the well-established pathway involving phosphoinositide (PI) hydrolysis by phospholipase C (PLC; reviewed by Loeffelholz, 1989; Exton, 1990; Billah & Anthes, 1990; Liscovitch, 1991). Yet the temporal and causal relationships of the two events remain uncertain. A possible experimental approach to this problem could involve the utilization of inhibitors that selectively inhibit either PLD or PLC, if such were available. Aminoglycoside antibiotics such as neomycin were shown to inhibit PI-PLC activity (Lipsky & Lietman, 1982; Schwert *et al.*, 1984) and have been utilized widely to probe the role of this enzyme in cell signalling. The mechanism of PI-PLC inhibition was presumed to result from the binding of the positively charged neomycin to the PIs which served as substrate for PLC. Indeed, whereas direct evidence *in vitro* for the binding of aminoglycosides to negatively charged phospholipids was obtained, no binding of neomycin and other aminoglycosides to PC could be observed (Sastrasinh *et al.*, 1982; Wang *et al.*, 1984). It was therefore reasonable to assume that neomycin would differentially inhibit PI-PLC activity without affecting PC hydrolysis by PLD. We now report that this is not the case, i.e. that neomycin and other aminoglycosides are potent inhibitors of a neutral PLD activity in permeabilized NG108-15 cells and in rat brain synaptic membranes.

METHODS

Materials

Bacterial PLD (from *Streptomyces chromofuscus*, type VI) and aminoglycoside antibiotics were obtained from Sigma. Guan-

osine 5'-[γ -thio]triphosphate (GTP[S]) was obtained from Boehringer-Mannheim. Sources of other materials were specified in our previous publications (Liscovitch, 1989; Liscovitch & Amsterdam, 1989; Chalifa *et al.*, 1990).

Cell culture and labelling

NG108-15 cells (passages 17–28) were routinely cultured as described previously (Liscovitch, 1989). Before experiments, cells were sub-cultured at a concentration of $(15\text{--}25) \times 10^3$ cells/cm² in 75 cm² flasks and grown to sub-confluence (usually 2–3 days). The medium was then replaced with Dulbecco's modified Eagle's medium containing 1 mg of fatty-acid-free BSA/ml (DMEM/BSA) and [3 H]oleic acid (50 μ Ci/75 cm² flask), and the cells were allowed to incorporate this precursor into membrane lipids for 20–24 h.

Assays of PLD activities

The activity of PLD in permeabilized NG108-15 cells was determined by measuring the accumulation of [3 H]phosphatidylethanol ([3 H]PEt) (specific product of the PLD transphosphatidyl transfer reaction) in cells that were pre-labelled with [3 H]oleic acid and permeabilized by digitonin. Briefly, NG108-15 cells were detached, suspended in DMEM/BSA (1×10^6 cells/ml) and allowed to recover for approx. 3 h at 37 °C. The cells were then washed by centrifugation in Dulbecco's phosphate-buffered saline (without Ca^{2+} and Mg^{2+}), and resuspended in buffer A, which contained: 20 mM-Na-Hepes, pH 7.2; 135 mM-KCl; 5 mM-NaHCO₃; 2.0 mM-MgCl₂; 1.5 mM-CaCl₂; 5 mM-EGTA; 5.6 mM-glucose; 2 mM-ATP (Mg^{2+} salt); 15 μ M-BSA; and 0.5% (v/v) ethanol (85 mM). The concentration of free Ca^{2+} was calculated to be 40 nM. The incubations were carried out in 12 mm $\times$ 75 mm glass tubes in a total volume of 0.4 ml at 37 °C. Incubations were started by adding the cell suspension (0.3 ml in buffer A) into tubes with 0.1 ml of buffer A containing digitonin (final concn. 20 μ g/ml) and the tested agent(s). Routinely, incubations were terminated after 30 min by adding 1.5 ml of ice-cold methanol/chloroform/conc. HCl/0.5 M-EDTA (200:100:2:3, by vol.) and vortex-mixing. Phase separation was accomplished by sequential addition of 0.5 ml of chloroform and 0.5 ml of 0.1 M-HCl. The

Abbreviations used: DMEM/BSA, Dulbecco's modified Eagle's medium containing 1 mg of fatty-acid-free BSA/ml; GTP[S], guanosine 5'-[γ -thio]triphosphate; PC, phosphatidylcholine; PEt, phosphatidylethanol; PI, phosphoinositide(s); PLC, phospholipase C; PLD, phospholipase D.

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lower chloroform phase was collected and evaporated by centrifugation under vacuum in a Speed-Vac concentrator. Lipid extracts were separated by t.l.c., and [^3H]PEt was quantified by liquid-scintillation spectrometry as previously described (Liscovitch & Amsterdam, 1989). The activity of PLD in rat brain synaptic plasma membranes was determined by measuring PET production from [^3H]dipalmitoyl-PC as described by Chalifa *et al.* (1990). The activity of *Streptomyces chromofuscus* PLD was determined essentially as described by Imamura & Horiuti (1979), with some modifications. Briefly, the reaction mixture contained (in a final volume of 0.4 ml): Tris/HCl, pH 8.0, 40 mM; CaCl_2 , 10 mM; [^3H]dipalmitoyl-PC, 2 mM (125 $\mu\text{Ci}/\text{mmol}$); PLD, 14.7 units (where 1 unit = 1 $\mu\text{mol}/\text{h}$). Incubations were carried out for 10 min at 37 °C. Termination and determination of [^3H]phosphatidic acid were as described by Chalifa *et al.* (1990).

RESULTS AND DISCUSSION

GTP[S] (25 μM) stimulated PLD activity about 6-fold in digitonin-permeabilized NG108-15 cells (Fig. 1); this action is MgATP- and Mg^{2+} -dependent, but Ca^{2+} -independent (M. Liscovitch & Y. Eli, unpublished work). The effect of neomycin on PLD activation by GTP[S] was examined by incubating permeabilized cells with increasing neomycin concentrations in the absence or presence of GTP[S] (Fig. 1). Neomycin inhibited GTP[S]-activated PLD-catalysed accumulation of [^3H]PEt in a dose-dependent manner, causing 50% inhibition at 100 μM and near-maximal inhibition at 700 μM . In contrast, basal PLD activity was not inhibited by neomycin; modest but reproducible stimulation of PLD activity (by 37%) was observed at 700 μM -neomycin (Fig. 1), and may be related to the ability of neomycin to activate G-proteins (cf. Aridor & Sagi-Eisenberg, 1990).

To determine whether the inhibitory effect of neomycin was due to inhibition of the PLD-catalysed reaction or to interference with PLD-G-protein coupling, we examined the action of several aminoglycosides on the activity *in vitro* of a neutral PLD that we have recently identified in rat brain synaptic plasma membranes (Chalifa *et al.*, 1990). This system provides the added advantage of employing a synthetic exogenous phospholipid ([^3H]dipalmitoyl-PC) as a substrate for PLD. Neomycin potently inhibited the rat brain PLD, causing 50% inhibition at 65 μM and 90% inhibition at 300 μM (Fig. 2). Other aminoglycosides tested, i.e. geneticin, kanamycin and streptomycin, were only slightly less effective than neomycin in inhibiting PLD activity. The relative similarity in the effectiveness of the four aminoglycosides tested stands in interesting contrast with their widely disparate potency in inhibiting PI-PLC, where neomycin is about 10-fold more potent than streptomycin (Table 1). In addition, the fact that neomycin (six amino groups) and geneticin (three amino groups) are nearly equipotent suggests that the positive charge density may not be a decisive factor in inhibiting PLD activity.

These results provide direct evidence that aminoglycosides inhibit PC-PLD activity at concentrations comparable with those shown previously to inhibit PI-PLC. The inhibition may result either from direct interaction of aminoglycosides with the enzyme protein, or from interaction with its phospholipid substrate PC, or with another phospholipid which might be required for its activity. Previous studies, employing various methodologies, have provided clear evidence that aminoglycosides bind to acidic phospholipids, particularly phosphoinositides, but not to PC (e.g. Sastrasinh *et al.*, 1982; Wang *et al.*, 1984). The possibility that aminoglycosides interacted with PC to inhibit PC-PLD activity was further excluded by demonstrating that neomycin had no effect on a PC-PLD activity isolated from *Streptomyces*

Fig. 1. Effect of neomycin on GTP[S]-stimulated PLD activity in permeabilized NG108-15 cells

Cells were incubated under routine assay conditions with increasing concentrations of neomycin, either without (○) or with (●) 25 μM -GTP[S]. [^3H]PEt production was measured as detailed in the Methods section. Values are means of duplicate determinations. Similar results were obtained in two other experiments.

Fig. 2. Effects of various aminoglycosides on synaptic membrane PLD activity

PLD activity was determined in the presence of increasing concentrations of neomycin (○), kanamycin (●), streptomycin (□) or geneticin (■). Results are expressed as percentages of activity obtained in the absence of inhibitor. Results represent the means of values from two to five independent experiments, each performed in duplicate.

Table 1. Comparison of the inhibitory potency of aminoglycoside antibiotics against various phospholipases

Abbreviation: N.D., not determined.

Aminoglycoside	Concn. causing 50% inhibition (mM)		
	PC-PLD*	PI-PLC†	PI-PLC‡
Neomycin	0.065	0.030	0.200
Geneticin	0.080	N.D.	N.D.
Kanamycin	0.125	0.300	2.700
Streptomycin	0.125	0.380	2.850
Gentamicin	N.D.	0.13	0.50
Tobramycin	N.D.	0.18	5.60
Amikacin	N.D.	0.34	8.85

* Brain membrane PC-PLD; values were estimated from Fig. 2.

† Kidney cytosolic PI-PLC; values are taken from Lipsky & Lietman (1982).

‡ Kidney membrane PI-PLC; values are taken from Schwertz *et al.* (1984).

chromofuscus (results not shown). Although aminoglycosides may interact directly with the PLD molecule, this possibility is inconsistent with our preliminary observations that solubilized brain PLD is not retained on an aminoglycoside affinity column (M. Liscovitch, M. Danin, unpublished work). Aminoglycosides may bind to vicinal (annular) acidic phospholipids and thus inhibit PLD activity, either simply by steric hindrance or because these acidic lipids are essential cofactors for PLD activity. Alternatively, aminoglycosides might stabilize the phosphatidyl-enzyme intermediate by binding to it and preventing its release from the catalytic site. Obviously, the elucidation of the molecular mechanism of PLD inhibition by aminoglycosides will require additional experimentation.

The present results underscore again (cf. Polascik *et al.*, 1987; Aridor & Sagi-Eisenberg, 1990) that caution should be exercised when utilizing aminoglycosides as inhibitors of PI-PLC activity. At the same time, PLD has been identified here as a possible pharmacological target for aminoglycoside action. The activation of PLD by receptor agonists has recently been recognized as an important novel signalling mechanism which is probably responsible for the sustained generation of the second messenger diacylglycerol in diverse cell types (Exton, 1990; Billah & Anthes, 1990). Further studies are required to examine the possibility that some of the toxic side effects associated with aminoglycoside treatment (e.g. ototoxicity, nephrotoxicity) involve its inhibitory effect on PLD activation.

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