Role of 2,6-Dideoxy-2,6-diaminoglucose in Activation of a Eukaryotic Phospholipase C by Aminoglycoside Antibiotics*

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Recent emergence of microbial resistance to aminoglycoside antibiotics, and the documented cytotoxicity associated with their use, calls for sustained efforts at understanding the effects of the compounds on eukaryotic cells. Using a glycosyl phosphatidylinositol (GPI)-phospholipase C (GPI-PLC) from the protozoan parasite Trypanosoma brucei, we demonstrate that a eukaryotic PLC can be activated 6-fold by aminoglycosides. Neomycin B protected GPI-PLC from a reduction in activity at pH 6.5, and increased the turnover number ($k_{cat}$) of the enzyme. In structure-activity studies with the neomycin group, 2-deoxy-streptamine was mildly stimulatory; the enzyme. In structure-activity studies with the neomycin group, 2-deoxy-streptamine was mildly stimulatory; the concentration required to activate GPI-PLC 2-fold ($SC_{200}$) was 310 $\mu$M. Neamine was 150-fold more active ($SC_{200} = 2$ $\mu$M) than 2-deoxy-streptamine, indicating that a 2,6-dideoxy-2,6-diaminoglucose substituent at the 4-position of 2-deoxystreptamine plays an important role in activation of GPI-PLC. Ribostamycin and neomycin B also had $SC_{200}$ of 2 $\mu$M, implying that the ribose group in ribostamycin is not involved in activation of GPI-PLC. These conclusions were affirmed in studies with Bacillus thuringiensis phosphatidylinositol-specific phospholipase C. A 2,6-dideoxy-2,6-diaminoglucose substitution at the 4-OH of 2-deoxystreptamine activates the enzyme 17-fold, while a second 2,6-dideoxy-2,6-diaminoglucose moiety on the ribose ring of ribostamycin provides an additional 3.5-fold stimulation. Possible implications of these observations for the effects of aminoglycosides on eukaryote cells are discussed.

Aminoglycosides have gained widespread use over the last fifty years as antibiotics against Gram-negative bacteria and some protozoa. In prokaryotes, aminoglycosides reduce the fidelity of protein synthesis on ribosomes and block polypeptide-tRNA translocation, through interactions with A-site 16S rRNA (1, 2) and elongation factor Tu (3). Recent emergence of drug resistance against aminoglycosides (4–6) suggests that efforts to understand all possible mechanisms of action of these compounds must be re-doubled with the long term aim of designing new and possibly more effective drugs.

Toxicity against mammalian cells (especially in the kidney) is one undesirable side effect of aminoglycoside antibiotics (reviewed (7)). Removal of this side effect from the next generation of aminoglycosides will require information on how these compounds affect mammalian cells.

Molecular geneticists employ aminoglycosides to eliminate eukaryote cells that fail take up and/or express selectable markers (e.g. neomycin phosphotransferase) after DNA-mediated transformation. Although the exact mode of cell killing has not been worked out, the antibiotics interfere with RNA splicing (8) and interact with negatively charged phospholipids (7). The toxic effects on eukaryote cells is thought to be due in part, at least, to alterations of the properties of the plasma and other cellular membranes (7).

Herein, we report stimulation of a purified eukaryotic enzyme, glycosyl phosphatidylinositol phospholipase C (GPI-PLC) from the protozoan parasite Trypanosoma brucei, itself susceptible to the antibiotics, by members of the neomycin group. Neomycin B protected the enzyme from a reduction in reaction rate observed at slightly acidic pH and increased the turnover number ($k_{cat}$) of GPI-PLC. Evidence is presented that 2,6-dideoxy-2,6-diaminoglucose in the neomycin group of aminoglycosides is very important for activation of the PLCs from T. brucei and Bacillus thuringiensis.

EXPERIMENTAL PROCEDURES

Materials—Recombinant GPI-PLC was purified by a published protocol (12). Purified PI-PLC from Bacillus thuringiensis was a gift from Dr. Ming-Daw Tsi (Ohio State University). 2-Deoxy-streptamine, neamine, and ribostamycin were gifts from Dr. Julian Davies (University of British Columbia). Geneticin (G418) was from Life Technologies, Inc. [9,10-3H]Myristic acid (40 Ci/mmol) was supplied by NEN Life Science Products. All other reagents, unless otherwise indicated were from Sigma. [3H]Myristate-labeled membrane-form variant surface glycoprotein (VSG) ([3H]mVSG) was purified from T. brucei (ILTat 1.3) as described previously (12).

Phospholipase C Assays—Reaction mixtures were assembled on ice in 1.5-ml microcentrifuge tubes. The quantity of enzyme used was 0.03 mg in 1.5 ml of 50 mM Hepes (pH 7.4) plus 100 mM NaCl, 10 mM MgCl$_2$, 100 mM KCl, 2 mM CaCl$_2$, 2 mM ATP, and 0.1% BSA. The reaction was started by the addition of 5-10 nmol of substrate. The reaction was stopped by the addition of 1.5 ml of an ice-cold 10% (v/v) Triton X-100 solution. This, with previous purification steps, was found to be adequate to remove all protein and substrate from the assay mixtures. The concentration of aminoglycoside required to activate an enzyme 2-fold; VSG, variant surface glycoprotein; PLC, phospholipase C; PI, phosphatidylinositol; PS, phosphatidylserine; SC$_{200}$, the concentration of aminoglycoside required to activate an enzyme 2-fold; VSG, variant surface glycoprotein; [3H]mVSG, [3H]myristate-labeled membrane-form variant surface glycoprotein.
range of the assay (13). GPI-PLC (9.5 units, 0.99 ng) was incubated with aminoglycoside antibiotics or control compounds in 20 μl of a mixed buffer (MES/Tris/CAPS, 50 mM final concentration) with 1% Nonidet P-40 for 10 min at 37 °C. Two μg of [3H]dimVSG in 10 μl of 1% Nonidet P-40 was added, followed by further incubation for 15 min at 37 °C. The reaction was terminated by chilling the mixture on ice and vortex mixing with 500 μl of water-saturated 1-butanol (at room temperature) to extract the cleaved [3H]dimyristoylglycerol. Phases were separated by centrifugation (12,000 × g, 1 min, 25 °C), and enzyme activity was quantified by measuring the amount of [3H]dimyristoylglycerol released into the upper butanol phase using a Beckman LS 6000TA scintillation counter. Activity of GPI-PLC obtained in the absence of test compounds was determined and assigned a value of 100%. Radioactivity from a mock digest of [3H]dimVSG (no enzyme added) was subtracted (as background) from all counts obtained.

Bacterial PI-PLC (0.03 ng, 1200 units/mg) was incubated with aminoglycoside antibiotics in a mixed buffer system (MES/Tris/CAPS, 25 mM final concentration) with 0.1% sodium deoxycholate for 10 min. [3H]dimVSG (in 0.1% sodium deoxycholate) was added, and product analysis was performed as described above for T. brucei GPI-PLC.

Effect of pH on Aminoglycoside Stimulation of GPI-PLC—The pH of the GPI-PLC assay (mixed buffer) was varied from pH 5.5 to pH 11.0. Buffer was added from a 500 mM stock (to 50 mM final concentration) with 1% (w/v) Nonidet P-40 (final concentration 1.0%) to 9.5 units of GPI-PLC. The mixture was incubated for 10 min at 37 °C with or without 0.3 mM neomycin B (from a 5 mM stock in H2O), followed by substrate addition, reaction initiation and termination as described above. (Purified substrate was in 1.0% Nonidet P-40 in H2O to avoid perturbing the pH of the reaction mixture.)

Determination of SC200—Varying amounts of aminoglycoside antibiotics were incubated at pH 6.5 for 10 min at 37 °C with GPI-PLC (9.5 units) or at pH 6 for PI-PLC (3.6 × 10−5 units), followed by addition of substrate and reaction completion as described above. The concentration of aminoglycoside antibiotics required to stimulate the phospholipases 2-fold (SC200) was obtained graphically (See Fig. 2).

RESULTS

Aminoglycosides Maintain Phospholipases C Activity at Sub-optimal pH—GPI-PLC is optimally active at about pH 9 (Fig. 1A). Enzyme activity is reduced 50% at pH 7.5 and 10, and nearly abolished at pH 6.5 or 11. In the presence of 0.3 mM neomycin B, the pH-activity profile of GPI-PLC is altered. The enzyme remains completely active at pH 7.5 and 60% active at pH 6.5, and neomycin B activated T. brucei GPI-PLC 6-fold as compared with a reaction in the absence of the aminoglycoside (Fig. 1). The pH optimum for the reaction decreased from pH 9.0 to 8.0 (Fig. 1). The activity of GPI-PLC under basic conditions is not altered by neomycin B (Fig. 1A). PI-PLC from B. thuringiensis is optimally active against a GPI at pH 7.0. Loss of activity below pH 7.0 was forestalled by addition of neomycin B (Fig. 1B).

Structure-Activity Relationship of Aminoglycoside Activation of PLC—To dissect features of aminoglycosides that influence phospholipase C activation, structurally related aminoglycosides from the neomycin group were examined (Fig. 2). 2-Deoxystreptamine stimulated GPI-PLC with a SC200 (the concentration of aminoglycoside required to activate the enzyme...
2-fold) of 310 μM (Fig. 2, inset, and Table I). Neamine (2-deoxystreptamine substituted at the 4 position with 2,6-dideoxy-2,6-diaminoglucose) was 150-fold more potent, with a SC\textsubscript{200} of 2 μM (Table I). Ribostamycin (neamine modified by the addition of a ribose) and neomycin B (ribostamycin with an additional 2,6-dideoxy-2,6-diamino-glucose group) were no more effective than neamine (SC\textsubscript{200} of 2 μM for both, Fig. 2 and Table I).

Non-glycosidic oligo or polyamines were tested. Putrescine (1,4-butanedianmine) had no effect on GPI-PLC at 0.3 mM. However, polymyxin B, spermidine [N-(3-aminopropyl)-1,4-butanedianmine], and polylysine (all at 0.3 mM), stimulated GPI-PLC 3.0-, 1.75-, and 2-fold, respectively (data not shown). We conclude from this set of observations that mere possession of amino groups is not sufficient to activate GPI-PLC. Possibly the active compounds adopt a “three-dimensional” conformation that is recognized by the enzyme.

*B. thuringiensis* PI-PLC was also activated by 2-deoxy-streptamine (SC\textsubscript{200} of 490 μM, Table I). Neamine was 17.5-fold more potent, with a SC\textsubscript{200} of 28 μM. Ribostamycin (SC\textsubscript{200} of 35 μM) was as potent as neamine, and neomycin B was 2.8-fold more active than neamine (SC\textsubscript{200} of 10 μM, Table I).

**Inhibition of GPI-PLC at pH 6.5 Is Prevented by Neomycin B**—After a 10-min incubation at pH 6.5, GPI-PLC is inhibited 90% as compared with enzyme that was kept at pH 9.0 (Figs. 1 and 3). In the presence of neomycin B (0.3 mM), enzyme activity is maintained even after a 15-min incubation in pH 6.5 buffer (Fig. 3). (The inhibition at the zero time point in the absence of neomycin B exists because the GPI cleavage assay is performed at pH 6.5 following the preincubation.) Neomycin B can rescue, at least partially, activity of GPI-PLC that has been preincubated at pH 6.5. Following a 5–15-min incubation at pH 6.5, neomycin B increased GPI-PLC activity 3–4-fold, when compared with an untreated control (Fig. 3). GPI-PLC activity is not altered by a 45-min incubation at 37 °C at pH 8.0 (data not presented).

**Acidic Phospholipids Influence Activation by Neomycin B**—Acid phospholipids inhibit GPI-PLC (13, 14), and additionally can interact with aminoglycosides (7). Either (or both) of these properties could reduce the extent of GPI-PLC activation by aminoglycosides *in vivo*. To test this hypothesis, we examined the effect of aminoglycosides on GPI-PLC in the presence of acidic phospholipids.

At pH 6.5, 5 mM PI had no effect on GPI-PLC, but PS (5 mM) strongly inhibited the enzyme (Fig. 4A). Neomycin B (0.3 mM) activated GPI-PLC 3-fold in the presence of PI (5 mM). As compared with the 6-fold stimulation obtained in the absence of PI, the neomycin B stimulation was reduced 50%. PS inhibition, however, was not altered by the presence of 0.3 mM neomycin B (Fig. 4A). Since the phospholipid concentration in these studies was in excess, we checked whether full stimulation by neomycin B would occur when the aminoglycoside was present at an equimolar concentration. Additional neomycin B (5 mM) stimulated GPI-PLC to extents similar to those of the control (*i.e.*, neomycin B with enzyme alone) (Fig. 4B). Similar results were obtained when these experiments were performed at pH 8.0, with the exception that PI now inhibited GPI-PLC as

| Antibiotic           | Structure | SC\textsubscript{200}^b | T. brucei GPI-PLC^b | B. thuringiensis PI-PLC^c |
|----------------------|-----------|-------------------------|---------------------|---------------------------|
| 2-Deoxystreptamine   | ![Structure](image) | 310                     | 490                 |                           |
| Neamine              | ![Structure](image) | 2                       | 28                  |                           |
| Ribostamycin         | ![Structure](image) | 2                       | 35                  |                           |
| Neomycin B           | ![Structure](image) | 2                       | 10                  |                           |

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^a SC\textsubscript{200} = aminoglycoside antibiotic concentration (μM) required to activate a phospholipase 2-fold.

^b GPI-PLC activity was assayed in 1.0% NP-40, 5 mM EDTA, 50 mM MES, pH 6.5.

^c PI-PLC was assayed in 0.1% sodium deoxycholate, 50 mM MES, pH 6.0.
previously reported (13).

We conclude from these observations that (i) aminoglycoside activation of GPI-PLC can occur in the presence of acidic phospholipids, that (ii) PI inhibition of GPI-PLC is pH-sensitive, but that (iii) inhibition by PS is not diminished by a reduction in the pH of the reaction.

**Neomycin B Alters the Kinetic Properties of PLCs**—Eadie-Hofstee analysis indicated that neomycin B at pH 6.5 increased the apparent turnover number ($k_{cat}$) of GPI-PLC 2.6-fold (Fig. 5, Table II), from 40 min$^{-1}$ to 102 min$^{-1}$. At pH 9.0, $k_{cat}$ of the enzyme increased to 144 min$^{-1}$, and neomycin B had no effect. Similarly, neomycin B did not change $k_{cat}$ at pH 10.5 ($k_{cat} = 117$ min$^{-1}$).

A 5.9-fold increase in the $K_m$ of GPI-PLC occurred with rising pH, from 75 nM at pH 6.5 to 445 nM at pH 10.5 (Fig. 5, Table II). In general, a slight decrease in $K_m$ was observed in the presence of neomycin B but the magnitude of the change was not dramatic.

Neomycin B affected *B. thuringiensis* PI-PLC differently. At pH 5.1, the apparent $K_m$ and $k_{cat}$ were 449 nM, and 231 min$^{-1}$, respectively (Table III). Addition of neomycin B caused an 8.9-fold increase in the apparent $K_m$ to 3980 nM, while the $k_{cat}$ increased 10.2-fold to 2349 min$^{-1}$. At pH 8.0, neomycin B again increased both the $K_m$ and $k_{cat}$ of PI-PLC significantly (7.7 and 5.7-fold, respectively) (Table III).

**DISCUSSION**

**Role of 2,6-Dideoxy-2,6-diamino-glucose in Activation of GPI-PLC by the Neomycin Group of Antibiotics**—The three major families of aminoglycoside antibiotics (*i.e.* the gentamicin (*e.g.* G418), kanamycin (*e.g.* tobramycin), and neomycin (*e.g.* neomycin B) groups) all contain 2-deoxy-streptamine. Existence of 4 structurally related members of the neomycin family enabled us to explore possible roles of the components of the antibiotics in activation of GPI-PLC. In the neomycin group (Table I) substitution of 2-deoxy-streptamine with 2,6-dideoxy-2,6-diamino-glucose produces neamine, which can be derivatized with ribose to form ribostamycin. Substitution of the ribosyl group in ribostamycin with 2,6-dideoxy-2,6-diamino-glucose generates neomycin B. Two PLCs in hand, one from a eukaryote *T. brucei* and the other from a prokaryote *B. thuringiensis*, one could explore the relative contributions of the chemical modifications of 2-deoxy-streptamine on activation of the phospholipases.

With both *T. brucei* GPI-PLC and *B. thuringiensis* PI-PLC, 2-deoxy-streptamine was the least effective at activating the GPI cleavage reaction (Table I). For GPI-PLC, introduction of the 2,6-dideoxy-2,6-diamino-glucose group (*i.e.* in neamine) onto this parent compound raised activation potency 150-fold. In comparison, *B. thuringiensis* PI-PLC was 17-fold more ac-
Ami-
glycosides May Contribute to Killing of Eukaryote Cells—
reduction in the fidelity of codon-anticodon interactions (17)
RNA splicing (8), binding to acidic phospholipids (16), and
effectiveness of the antibiotics. In eukaryotes, inhibition of
the genetic code due to aminoglycoside occupancy of the A-site
are used to treat infections caused by Gram-negative bacteria
Ami-
glycosides with cellular phospholipids have not focused on
possible downstream effects following the interaction of ami-
glycosides with cellular phospholipids have not focused on
membrane bound proteins. These antibiotics interact with eu-
karyote 18 S rRNA (17) and phospholipids (7). Direct binding to
eukaryote proteins as a possible contributor to the development
of cytotoxicity/nephrotoxicity during administration of these
compounds has received little attention for lack of evidence.
Because a eukaryotic GPI-PLC can be stimulated by several
aminoglycosides (Table II), the possibility that aminoglycosides
exert their effects in part by modulating the activity of cellular
phospholipases merits serious consideration, chiefly because
persistent activation of a cellular phospholipase C, especially
under conditions when one had expected the enzyme to be
inactive, could have negative consequences on cellular physiol-
yology. In cells that are known to accumulate the antibiotics to
millimolar concentrations in acidic compartments (e.g. kidney
cells (7) and fibroblasts (18)) the situation might be even more
alarming.

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