Inhibition of in Vitro Endosomal Vesicle Fusion Activity by Aminoglycoside Antibiotics

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The effects of two aminoglycoside antibiotics, neomycin and Geneticin, on the endocytic pathway were studied using a cell-free assay that reconstitutes endosome-endosome fusion. Both drugs inhibit the rate and extent of endosome fusion in a dose-dependent manner with IC₅₀ values of ~45 μM and ~1 mM, respectively. Because the IC₅₀ for neomycin falls within the range of affinities reported for its binding to acidic phospholipids, notably phosphatidylinositol 4,5-bisphosphate (PIP₂), these data suggest that negatively charged lipids are required for endosome fusion. A role for negatively charged lipids in membrane traffic has been postulated to involve the activity of a PIP₂-dependent phospholipase D (PLD) stimulated by the GTP-binding protein ADP-ribosylation factor (ARF). Although neomycin blocks endosome fusion at a stage of the in vitro reaction that is temporally related to steps inhibited by cytosolic ARFs when they bind guanosine-5′-γ-thiophosphate (GTPγS), these inhibitors appear to act in a synergistic manner. This idea is confirmed by the fact that addition of a PIP₂-independent PLD does not suppress neomycin inhibition of endosome fusion; moreover, in vitro fusion activity is not affected by the pleckstrin homology domain of phosphoinositide-specific phospholipase C δ1, which binds to acidic phospholipids, particularly PIP₂, with high affinity. Thus, although aminoglycoside-sensitive elements of endosome fusion are required at mechanistic stages that are also blocked by GTPγS-bound ARF, these effects are unrelated to inhibition of the PIP₂-dependent PLD activity stimulated by this GTP-binding protein. These results argue that there are additional mechanistic roles for acidic phospholipids in the endosomal pathway.

Activation of phospholipase D (PLD)† by the GTP-binding protein ADP-ribosylation factor (ARF) is theorized to be a pivotal event in membrane trafficking (1–4). ARF-activated PLD mediates the hydrolysis of phosphatidylcholine (PC) to phosphatic acid (PA) in a manner dependent on phosphatidylinositol 4,5-bisphosphate (PIP₂) (3, 5–7). PA, the product of PLD’s action, in turn activates phosphatidylinositol 4-phosphate 5-kinase, thereby stimulating a positive feedback cycle as the latter enzyme generates PIP₂ (8, 9). It has been proposed that the local production of PA and PIP₂ facilitates the recruitment of coatamer subunits (collectively referred to as the COPI coat) onto Golgi membranes (4). This idea is consistent with the documented role of ARF in COPI-mediated vesicle traffic in the secretory pathway (reviewed in Ref. 1). PLD activation by ARF appears to stimulate the budding of secretory vesicles from the trans-Golgi network as well (10, 11), suggesting that transient changes in lipid composition promoted by ARF may also recruit clathrin and its associated complex, AP-1, to generate clathrin-coated vesicles. In fact, GTPγS-bound ARF is known to induce the association of AP-1 adapters with trans-Golgi network membranes and immature secretory granules (12–15), and these findings may reflect biochemical events preceding co-assembly of clathrin into nascent clathrin-coated buds (13).

Although increasing evidence to support a role for ARF activation of PLD activity in the exocytic pathway has emerged, relatively little information is available regarding the function of these elements in the endocytic pathway. Recently, Robinson and colleagues (16, 17) have described the recruitment of the clathrin-associated AP-2 adaptor complex onto endosomal membranes in response to GTPγS-binding by cytosolic ARFs. This group of investigators demonstrated that addition of exogenous PLD had similar effects; hence, the activation of endogenous PIP₂-dependent PLD by ARF is likely to be responsible for AP-2 recruitment. Although these results point to a functional role for ARF-dependent recruitment of AP-2 to support endosomal membrane trafficking, in vitro studies from our laboratory indicate that soluble ARF activity is not required for endosome-endosome fusion despite the fact that cytosolic ARFs inhibit this activity when bound to GTPγS (18). Nonetheless, PLD has been implicated to function in endosome fusion; an inhibitor of PA production by PLD, 1-butanol, suppresses in vitro fusion activity, whereas exogenously added PLD can stimulate this reaction (19).

To better understand the role of ARF and PLD in the endocytic pathway, we chose to investigate the effects of aminoglycoside antibiotics. These agents are known to bind to negatively charged lipids (reviewed in Ref. 20); in particular, it has been demonstrated that neomycin specifically interacts with the acidic phospholipid PIP₂ (see Ref. 21 and references therein). Neomycin inhibits PIP₂-dependent PLD (3), and interferes with AP-2 recruitment onto endosomes (17). Neomycin also has been used in studies of an ARF-regulated PLD activity that appears to be involved in neutrophil activation (22) and an ARF-regulated PLD activity that enhances release of secretory vesicles from the trans-Golgi network (10); moreover, a second aminoglycoside, Geneticin, has been shown to block secretory vesicle formation stimulated by exogenous PLD (11).
Aminoglycosides also have been widely studied because of the nephrotoxicity associated with their use as antibiotics and are well known to cause a vacuolarization of the apical endosomal compartment of renal proximal tubules (23, 24). Furthermore, Giurgea-Marion et al. (25) have shown that the aminoglycoside gentamicin prevents the delivery of herderidaz peroxidase to the lysosome, implicating a function for acidic lipids in endocytic membrane traffic. Although many investigators have attributed the nephrotoxicity of aminoglycosides to steps affected by GTPase inhibitors of cell-free endosome-endosome fusion with IC50 values consistent with their known binding interactions with acidic phospholipids. Our data demonstrate that these aminoglycosides block steps of endosome fusion temporally related to steps affected by GTP-γS-bound ARFs; however, the fact that these antibiotics act synergistically with GTP-γS to inhibit fusion suggests that additional endosomal functions are affected. This concept is supported by the observations that a PI-PLC- independent PLD does not suppress the effects of neomycin on endosome fusion and that this in vitro activity is unaffected by the pleckstrin homology (PH) domain of phosphoinositide-specific phospholipase C-β1 (PI-PLCβ1), which is known to bind PI(3,4,5)P3 with high affinity (26, 27). These results argue that there are additional mechanistic roles for acidic phospholipids in the endosomal pathway.

**EXPERIMENTAL PROCEDURES**

**Preparation of K562 Cell Cytosol and Membrane Fractions—**K562 cells were maintained in a minimal essential medium supplemented with 7.5% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Post-nuclear supernatant (PNS) fractions were prepared as described previously (18). Briefly, K562 cells were washed in phosphate-buffered saline (PBS), resuspended in 25 mM Hepes, pH 7.5, 150 mM NaCl, 1 mg/ml dextrose, 1 mg/ml bovine serum albumin, and incubated with either 0.5 mg/ml aminoglycoside or 100 nM biotin-transferrin for 60 min at 20 °C. After endocytosis was quenched and ice, the cells were broken and washed using a ball bearing homogenizer in breaking buffer (20 mM Hepes, pH 7.4, 100 mM KCl, 85 mM sucrose, 20 μM EGTA). PNS was collected upon centrifugation at 800 × g for 5 min at 4 °C, and after dialysis against breaking buffer, aliquots were stored frozen at −80 °C until use. Cytosol was prepared from K562 cells washed in PBS and broken in the same buffer, except that, after the post-nuclear centrifugation step, the PNS was subjected to ultracentrifugation for 15 min at 350,000 × g at 4 °C to remove membranous components. Supernatant cytosol was also dialyzed against breaking buffer and stored frozen at −80 °C. Endocytic Vesicle Fusion Assay—**In vitro endosomal fusion assays were carried as described by Wessling-Resnick and Braeul (28). PNS fractions with endocytic vesicles containing avidin-β-galactosidase and biotin-transferrin were mixed on ice in a buffer containing cytosol, 1 mM MgATP, 50 μg/ml creatine kinase, 0.8 mM phosphocreatine, 10 μg/ml biotin-insulin, and 1 mM dithiothreitol. For some experiments, vesicles were first collected by microcentrifugation at 13,000 × g for 5 min at 4 °C prior to the fusion assay. Details of the amount of cytosol in the assay mixture, the exact manipulation of PNS fractions, and conditions of treatment with aminoglycoside antibiotics are found in the figure legends; all manipulations of cytosol at 37 °C prior to fusion assays were carried in the presence of ATP, an ATP-regenerating system, and 1 mM dithiothreitol as indicated above. Reaction mixtures were held at 37 °C for the times indicated in the figure legends, and fusion was terminated by 1:10 dilution of lysis buffer (10% Triton X-100, 1% sodium dodecyl sulfate, 50 μg/ml biotin-insulin). After microcentrifugation, clarified lysates were placed in a microtiter wells coated with anti-transferrin antibodies (1:100 dilution).

The complex between avidin-β-galactosidase and biotin-transferrin resulting from specific fusion events was measured as described previously (29). Briefly, after overnight incubation of the lysates at 4 °C to capture the avidin-β-galactosidase/biotin-transferrin complex, microtiter wells were washed three times with PBS, followed by four washes with 10 mM Tris, pH 7.5, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 100 mM NaCl, 1 mM EDTA. The wells were incubated 20 min at 37 °C in a final wash, then rinsed with PBS three times. Avidin-β-galactosidase activity was measured by incubating the wells with 250 μl of swelling solution (0.3 mM MgATP, 20 μg/ml phosphodiesterase, 100 mM NaCl, 25 mM Tris, pH 7.4, 1 mM MgCl2, 12 μM β-mercaptoethanol) for 1–3 h. Samples were diluted with five volumes of 133 mM glycine, 83 mM Na3CO3, pH 10.7, and the fluorescence of the hydrolysis product was measured using an Hitachi F-2000 spectrophotometer (365 nm excitation, 450 nm emission). The fluorescence signal is directly proportional to the extent of probe colocalization detected by complex formation (28, 29). Fusion measurements were adjusted for background by subtracting the amount of activity measured in samples incubated at 4 °C; because of the observed effects of neomycin and Genetin to precipitate proteins (see "Results"), care was taken to precisely match 4 °C control samples that included the appropriate concentration of antibiotic. Fusion activity is presented as the amount of activity measured relative to control reactions carried out in the absence of drug.

**Phospholipase D Assay—**PLD assays were performed using methods established by Danin et al. (30). Briefly, aliquots of 1-palmitoyl-2-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]caproyl-sn-glycerol-3-phosphocholine (C12-NBD-PC) in chloroform were dried under nitrogen and dissolved in 20 mM HepES, pH 7.4, 85 mM sucrose, 150 mM KCl upon sonication for 5 min. Assay mixtures (41 μl final volume) contained 3 μl of 100 μM NBD-PC, 10 μl of PNS, 20 μl of cytosol (11.7 μg/ml), and the ATP-regenerating system as described for endosome fusion assays. PLD was added from a 50 units/μl stock as noted in the figure legends. After a 1-h incubation at 37 °C, lipids were extracted into chloroform, lyophilized, resuspended in 50:50 chloroform:methanol, and then spotted on PEK Silica gel 60 plates (Whatman). The thin layer chromatography plates were developed in 2-propanol:ethyl acetate:chloroform:water (50:50:20:18, v/v). The resolved fluorescent lipids were visualized by UV light with C12-NBD-PC and 1-palmitoyl-2-[(7-nitro-2-1,3 benzoxadiazol-4-yl)amino]caproyl-sn-glycerol-3-phosphate (C12-NBD-PA) used as standards.

**Materials and Miscellaneous Methods—**Avidin-β-galactosidase, biotin-transferrin, biotin-insulin, polyaspartic acid (10.4 kDa), Streptomyces plasmatosurinum PLD (200 units/mg), and neovin sulfate were from Sigma; Genetin (G418) was purchased from Life Technologies, Inc. C12-NBD-PC and C12-NBD-PA were obtained from Avanti Polar Lipids. Anti-transferrin antibodies were from Research Diagnostics. PI-PLCβ1 and PI-PLC61A (1–132), which lacks the phospholipase PH domain, were a generous gift of Dr. Mary Roberts (Department of Chemistry, Boston College, Boston, MA). SDS-polyacylamide gel electrophoresis was performed according to Laemmli (31), and protein concentration was determined by the Bradford assay (32) using bovine serum albumin as a standard.

**RESULTS**

To characterize the effects of neomycin, endosome-endosome fusion activity was measured in cell-free assays supplemented with increasing amounts of drug. Briefly, vesicles containing endocytosed biotin-transferrin or avidin-β-galactosidase were mixed together on ice with cytosol, ATP, an ATP-regenerating system, and biotin-insulin; the latter is added to scavenge any extravascular avidin-β-galactosidase that would otherwise create an artificial fusion signal. When incubated at 37 °C, fusion of endosomal compartments results in colocalization of the probes (28); the amount of complex formed (biotin-transferrin: avidin-β-galactosidase) is quantified using a modified enzyme-linked immunosorbent assay format and is directly proportional to the extent of fusion activity in the assay (29). As demonstrated by results shown in Fig. 1, neomycin inhibits cell-free endosome fusion in a dose-dependent manner with an IC50 of 45 μM.

Recent studies by Hudson and Draper (33) revealed that neomycin specifically blocks binding of the soluble COPI complex to Golgi membranes. Thus, the effect is a result of the precipitation of customer with half-maximal effects observed at ~50 μM neomycin. Thus, a plausible explanation for the dose-response curve shown in Fig. 1 is that the drug precipitates or inactivates COPI subunits that might mediate steps in the process of endosome fusion. COPI proteins have been localized to endosomal membranes (34, 35), and Chinese hamster ovary
cell IdIF mutants have a temperature-sensitive mutation in e-COP that causes defects in endocytic membrane traffic (36). Furthermore, although evidence to support a role of COPI proteins in early endocytic vesicle fusion events is lacking, Aniento et al. (35) have demonstrated that bCOP is required for the in vitro formation of endosomal carrier vesicles that support transport from early to late endocytic compartments. To examine whether inhibition by neomycin might be exerted as a result of a COPI coat requirement in the fusion assay, the effects of a second aminoglycoside, Geneticin (G418), were studied. Geneticin fails to precipitate coatomer, although this drug can block bCOP binding to Golgi membranes (33). Similar to neomycin, Geneticin inhibits in vitro fusion activity in a dose-dependent manner (Fig. 2). However, the IC50 for Geneticin inhibition (~1 mM) is roughly an order of magnitude less than that observed to block coatomer-membrane interactions (33). Because Geneticin does not precipitate coatomer or prevent its binding to membranes over the range of the inhibitory concentrations observed in our experiments, it is unlikely that the aminoglycosides block endosome fusion as a result of the precipitation and/or inactivation of COPI proteins. Nevertheless, a precipitate was observed to form when cytosol was treated with higher concentrations of neomycin or Geneticin. SDS-polyacrylamide gel electrophoresis of these precipitates reveals a number of Coomassie-stainable bands (Fig. 3). These observations extend the results of Hudson and Draper (33) to demonstrate that many soluble factors can be precipitated by both drugs in addition to COPI subunits. As shown in Fig. 4, neomycin causes precipitation of total protein with an EC50 ~ 250 μM (top panel), whereas Geneticin is less effective with an EC50 > 2 mM (bottom panel). The relative activities of the two antibiotics to cause precipitation corresponds to the number of amino groups they contain (6 and 3, respectively), suggesting that these polyaminated drugs may nonspecifically interact with acidic proteins to promote their denaturation.

Because of our concern that inhibition of fusion activity might arise solely from effects caused by protein precipitation, the ability of neomycin- and Geneticin-treated cytosol to support fusion activity was studied. Prior to fusion assays, the precipitate resulting from incubation with these drugs (approximately 1–2% of total protein) was removed by centrifugation, and the clarified cytosol was passed over a Sephadex G-25 spin column to remove antibiotic. The clarified and gel-filtered cy-
tosol was then examined for the capacity to support fusion activity with membranes separated from PNS fractions. Removal of the drugs and the elimination of precipitated proteins restored vesicle fusion; as shown in Fig. 5, neomycin- and Geneticin-treated cytosol supported fusion activity to 65% and 90% of control levels, respectively. Addition of neomycin or Geneticin back to the pretreated cytosol inhibited endosome fusion (25% of control levels), verifying that an aminoglycoside-sensitive factor is indeed present on endosomal membranes. Nonetheless, under the conditions defined by this assay, unspecific precipitation and/or irreversible inactivation of cytosolic protein(s) by the antibiotics does appear to have modest effects on fusion activity. This conclusion is consistent with the fact that the extent of endosome fusion activity is generally dependent on cytosol concentration (18, 28, 37), although the possibility that a specific cytosolic fusion factor is targeted by these drugs cannot be rigorously excluded. Because of the pleotropic nature of the effects exerted by neomycin and Geneticin, we elected to continue our investigation using the conditions carefully defined by these control experiments (≤250 μM neomycin and 2 mM Geneticin). These concentrations are less than or equal to the apparent EC50 for protein precipitation but are 2–5-fold higher than half-maximal concentrations for inhibition of fusion activity. Thus, depletion of cytosolic factors owing to precipitate formation are minimized whereas inhibitory effects on membrane components are maximized.

To exclude the possibility that the aminoglycoside antibiotics might act on the endosomal bilayer to cause detergent-like effects, we studied the ability of polyaspartic acid to block the action of the drugs on fusion activity. Polyaspartic acid is known to bind and displace the aminoglycoside antibiotic gentamycin from negatively charged liposomes and purified membrane vesicles (23). As might be predicted, this homopolymer also effectively suppresses inhibition of endosome-endosome fusion activity by neomycin and Geneticin (Fig. 6). These results argue that the aminoglycosides do not simply disrupt the endosomal bilayer in a detergent-like fashion, but rather that the antibiotics’ effects are electrostatic in nature. In additional control experiments, PNS fractions were incubated with 100 μM neomycin or 2 mM Geneticin at 4 °C, and treated membranes were then separated by centrifugation, washed to remove residual antibiotic, and assayed for fusion activity (Fig. 7). The fact that nearly 100% of control fusion activity is observed for neomycin- and Geneticin-treated membranes is entirely consistent with the notion that the antibiotics bind to membrane components, but do not disrupt the bilayer to inhibit endosome fusion.

To investigate whether aminoglycosides alter the rate and/or extent of vesicle fusion, time-course experiments were performed. The effects of 100 μM neomycin (solid circles) and 2 mM Geneticin (open squares) on fusion activity as a function of time is shown in Fig. 8. Both aminoglycosides reduce the rate and extent of vesicle fusion, but these results suggest that some degree of activity present in the assay system is resistant to inhibition by the aminoglycosides. This portion of fusion activity may represent “primed” components that are ready to participate in bilayer fusion and therefore have proceeded past stages in the reaction mechanism affected by the drugs (see below). These observations further argue that the drugs do not...
perturb the integrity of the membrane bilayer to inhibit fusion activity.

The observed IC₅₀ for neomycin correlates quite well with the drug’s reported affinities for anionic phospholipids (10–100 μM) (38). Because of the relative specificity of neomycin for PIP₂ binding (39), and the fact that this lipid cofactor is required for ARF-stimulated PLD activity (3–7), we next explored the relationship between aminoglycoside- and GTP₇S-sensitive steps of endosome fusion. We (18, 28) and others (40) have demonstrated that when activated by GTP₇S, cytosolic ARFs will inhibit endosome fusion. To examine the temporal steps of endosome fusion affected by neomycin and GTP₇S, cell-free assays were carried out at 37 °C in the absence of added inhibitor until the times indicated in Fig. 9. At these time points, either neomycin was directly added to the reaction mixture or endosomal membranes were separated and resuspended in new assay mixtures containing cytosol treated with GTP₇S to activate ARFs. As expected, the ability of neomycin or GTP₇S to inhibit vesicle fusion decreases with time. However, resistance to both inhibitors appears more or less about the same time; within approximately 10–15 min, reaction intermediates appear that are no longer sensitive to inhibition. This observation demonstrates that aminoglycosides and cytosolic ARFs inhibit an early stage of the fusion reaction, and is consistent with our previous data showing that GTP₇S blocks steps preceding N-ethylmaleimide-sensitive steps of endosome fusion (37). Similar results were obtained with Geneticin (data not shown).

One interpretation of the effects of GTP₇S-bound ARF is that activation of PLD, which would be stimulated under these conditions (5–7), interferes with endosome fusion. This model presumes that the PLD activity implicated in endosome fusion
Aminoglycosides Inhibit Endosome Fusion

Fig. 10. Aminoglycosides potentiate inhibition of endosome fusion by GTPγS. Fusion assay mixtures containing 6.8 mg/ml cytosol and 2.5-μl aliquots of each PNS containing avidin-β-galactosidase and biotin-transferrin were prepared and incubated for 30 min at 37 °C with 100 μM neomycin (open bars), 2 mM Geneticin (hatched bars), or no drug (solid bars) in the presence (+) or absence (−) of GTPγS. The reactions were quenched on ice, and membranes were isolated by centrifugation and then assayed for fusion activity upon addition untreated cytosol, ATP, and an ATP-regenerating system as described for Fig. 1.

(19) would ordinarily function in a manner regulated by membrane-associated factors as depletion of cytosolic ARFs does not affect fusion activity but eliminates the inhibitory effects of GTPγS (18). If ARF or an ARF-like activity associated with endosomes is involved in PLD activation, then the binding of PIP2 by neomycin might interfere with its GDP/GTP exchange (41, 42), the activity of the phospholipase itself, which requires PIP2 as a cofactor (3), or the hydrolysis of GTP stimulated by ARF-GTPase-activating factor, which is enhanced by acidic phospholipids (43). All of these scenarios are compatible with the temporal relationship between neomycin and GTPγS inhibition established by results shown in Fig. 9. A prediction of this model is that neomycin would block the inhibitory effects of GTPγS-bound ARFs such that fusion activity might be recovered when both inhibitors are removed from the assay system.

To test the hypothesis that aminoglycosides would interfere with ARF function to block inhibition of endosome fusion by GTPγS, a preincubation step was carried out with assay mixtures containing either 100 μM neomycin or 2 mM Geneticin and cytosol treated with or without GTPγS (Fig. 10). Membranes were then separated and resuspended in new reaction mixtures containing untreated cytosol, ATP, and an ATP-regenerating system to assay fusion activity. Previous studies have shown that GTPγS inhibition of endosome-endosome fusion (18, 28) and intra-Golgi transport (44) is irreversible, consistent with control measurements in this experiment (solid bars). Elimination of the aminoglycosides from samples that were treated with neomycin (open bars) and Geneticin (hatched bars) alone partially restored fusion. Although this result is consistent with the observed inactivation and/or precipitation of cytosolic components (Fig. 5), the extent of activity recovered is less than expected. Because treatment of membranes at 4 °C does not alter fusion (Fig. 7), the additional loss of activity upon incubation at 37 °C in the presence of ATP suggests that the aminoglycosides may affect dynamic membrane processes to inactivate endosome fusion. Nonetheless, the residual fusion activity measured after elimination of the drugs is completely abolished when samples were co-incubated with GTPγS. Thus, the aminoglycosides do not prevent the inhibitory effects of GTPγS-bound ARFs; rather, the antibiotics appear to act in a synergistic manner to block endosome fusion.

To confirm that aminoglycosides influence functions other than the PIP2-dependent PLD activity to block endosome fusion, the ability of a PIP2-independent PLD to suppress the effects of neomycin was evaluated. As shown in Fig. 11 (lower panel), addition of 0.1 unit/μl S. chromofuscus PLD and 100 μM neomycin as indicated. Data represent the mean (± S.D.) from two identical experiments. Lower panel, PLD assays containing 200 μM C6-NBD-PC in PNS with 4.1 mg/ml cytosol were incubated for 30 min at 37 °C and resulting products were separated by thin layer chromatography as described under “Experimental Procedures.” These conditions were the same as those used in fusion assay experiments shown in the upper panel, with 0.1 unit/μl S. chromofuscus PLD, 200 μM neomycin and/or 150 μM GTPγS added as indicated; control samples held at 4 °C are shown in lane 3. Lanes 1 and 2 show the migration positions of NBD-PC and NBD-PA standards, respectively.

GTPγS-bound ARFs; rather, the antibiotics appear to act in a synergistic manner to block endosome fusion.

A second observation from these experiments is that, whereas GTPγS stimulates PC hydrolysis in our assay system, neomycin has very modest effects at concentrations found to block endosome fusion (Fig. 11, lower panel). This result is consistent with previous observations reported by West et al. (17). Using permeabilized cells, the latter group found that addition of GTPγS stimulated basal PLD activity ~30%, but

FIG. 11. Neomycin inhibits fusion in the presence of active exogenous PLD. Upper panel, fusion assays were carried out as in Fig. 1 but contained 16 μM CaCl2 and were incubated with or without 0.1 unit/μl S. chromofuscus PLD and 100 μM neomycin as indicated. Data represent the mean (± S.D.) from two identical experiments. Lower panel, PLD assays containing 200 μM C6-NBD-PC in PNS with 4.1 mg/ml cytosol were incubated for 30 min at 37 °C and resulting products were separated by thin layer chromatography as described under “Experimental Procedures.” These conditions were the same as those used in fusion assay experiments shown in the upper panel, with 0.1 unit/μl S. chromofuscus PLD, 200 μM neomycin and/or 150 μM GTPγS added as indicated; control samples held at 4 °C are shown in lane 3. Lanes 1 and 2 show the migration positions of NBD-PC and NBD-PA standards, respectively.
that this activity was only inhibited by ∼40% at 10-fold higher levels of neomycin than those used in our experiments. These findings were interpreted to reflect additional PLD isoforms that are neomycin-insensitive. This conclusion is consistent with the levels of GTPγS-stimulated PA production shown in Fig. 11, which provide an additional indication that inhibition of a neomycin-sensitive PLD is unlikely to account for the effects of aminoglycosides on endosome fusion. Although it is possible that the minor fraction of neomycin-sensitive PLD activity observed upon GTPγS stimulation is directly associated with endosomal vesicles participating in fusion events, the hypothesis that the effects of aminoglycosides on membrane traffic go beyond inhibition of the ARF-activated PIP2-dependent PLD is substantially supported by our observation that a PIP2-independent PLD fails to suppress the effects of neomycin (Fig. 11), as well as the findings that neomycin inhibition of ARF-stimulated AP-2 recruitment to endosomes (17) and inhibition of secretory vesicle biogenesis by Geneticin (11) also cannot be reversed by addition of exogenous PLD.

Although the experimental results presented in Fig. 11 argue against ARF-regulated PLD as a sole target for neomycin inhibition, they do not exclude the possibility that the aminoglycosides interfere with other PIP2-dependent activities potentially involved in vesicle fusion as a result of their interaction with this acidic lipid. High affinity binding interactions of the PH domain of PI-PLCΔ with PIP2 are also well described (26, 27). Therefore, we utilized purified PI-PLCΔ and a truncation mutant lacking the PH domain, PI-PLCΔ (1–132), to study the effects of a second PIP2-binding reagent on endosome fusion activity. As summarized by the results shown in Fig. 12, at concentrations as high as 1 μM, PI-PLCΔ did not influence endosomal vesicle fusion and did not reverse neomycin inhibition of this activity; the catalytically active PH truncation mutant was also without effect. The fact that this PIP2-binding protein does not interfere with fusion activity is a second indication that neomycin inhibition entails more than the simple sequestration of PIP2 to prevent its utilization as either a substrate or cofactor for phospholipases; rather, this finding suggests additional roles for acidic phospholipids in the mechanism of endosome fusion.

DISCUSSION

Our investigation demonstrates that neomycin and Geneticin inhibit in vitro endosome fusion in a dose-dependent manner; IC50 values of ∼45 μM and ∼1 mM were determined, respectively. These data further reveal that the antibiotics may exert their effects in a pleiotropic manner because of their capacity to precipitate cytosolic proteins as well as to interact with endosomal membranes. Hudson and Draper (33) have shown that neomycin can interfere with recruitment of coatomer to membranes as a result of precipitation of COPI proteins with an IC50 ∼ 50 μM. Because Geneticin, an aminoglycoside that fails to precipitate coatomer, inhibits our fusion assay under conditions wherein coatomer recruitment is unlikely to be perturbed, endosome fusion does not appear to be blocked because of effects on COPI function. This conclusion is supported by ongoing studies in our laboratory that indicate depletion of cytosolic COPI proteins does not alter endosome fusion activity.2 Furthermore, although a role for COPI proteins in early-to-late endosome fusion has been defined, evidence to support a function in early endosome fusion is lacking (35). Although neomycin and Geneticin do promote the precipitation of other soluble proteins, most likely via ionic interactions with the polyaminated antibiotics (45), the following lines

of evidence argue that the primary target for inhibition of endosome fusion is a membrane factor. 1) the IC50 values observed for total protein precipitation are much higher than IC50 values determined for aminoglycoside inhibition; 2) the IC50 for neomycin falls within the range of affinities determined for binding to acidic phospholipids (38); and 3) although elimination of the protein precipitate causes some loss in endosome fusion activity, readdition of the drug with membranes completely abolishes this residual activity (Fig. 5).

A major finding in our study is that the temporal requirement for the aminoglycoside-sensitive function in cell-free endosome fusion is coincident with GTPγS-sensitive stages of this reaction. We initially elected to study the effects of aminoglycoside antibiotics to explore the potential role of PIP2-dependent PLD activity stimulated by GTPγS-bound ARF in the endocytic pathway. We (18) and others (40) have previously reported that GTPγS-bound ARF inhibits endosome fusion and recent studies have highlighted a role for ARF-stimulated PLD activity in membrane traffic events involving the recruitment of coat proteins and the production of vesicles in the secretory

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pathway (10, 11, 16, 17). If chronic activation of PLD by GTPγS-bound ARFs is responsible for inhibition of endosome fusion, then neomycin would be predicted to reverse this effect because of its ability to block the activity of the PIP$_2$-dependent PLD (3). Instead, our experiments clearly demonstrate that aminoglycosides potentiate GTPγS inhibition in a synergistic fashion. Furthermore, addition of exogenous PIP$_2$-independent PLD does not suppress inhibition of endosomal vesicle fusion by neomycin, confirming that this drug must target mechanistic functions that are unrelated to PLD activation but that are required for fusion activity; the observation that neomycin only marginally inhibits endogenous GTPγS-stimulated PLD activity in our assay system is consistent with this conclusion. In fact, neither neomycin inhibition of AP-2 recruitment to endosomes (17) nor inhibition of secretory vesicle biogenesis by Gentamicin (11) can be reversed upon addition of exogenous PLD. These observations further support our hypothesis that the effects of aminoglycosides on membrane traffic are independent of the PLD activity stimulated by ARF.

It is important to note that the aminoglycoside antibiotics could potentially interact with a protein factor required for endosomal vesicle fusion. Because the effects on vesicular components are readily reversible, it is unlikely that the aminoglycosides simply denature functionally relevant acidic membrane protein(s); instead, these drugs appear to bind their membrane-associated target in a reversible manner. The precise temporal nature of the drugs’ effects during the progress of endosome fusion further argues for the requirement of a highly specific neomycin-sensitive function at a time when the reaction becomes most susceptible to inhibition by GTPγS. Hammond et al. (46) reported that the aminoglycoside gentamicin inhibits fusion between isolated renal apical endosomes in vitro and assigned these effects to the binding of the drug to the membrane protein gp330/megalin. Although there is evidence to indicate that aminoglycosides do indeed interact with the gp330/megalin receptor (47), its tissue-restricted expression argues against the possibility that this particular protein factor is involved in our endosome fusion assay system. It is quite likely that the aminoglycoside effects witnessed in this earlier study are more related to the inhibition of endosome fusion by neomycin and Gentamicin revealed by our investigation rather than indirect action because of the internalization of gentamycin with the gp330/megalin receptor. However, we are unaware of any other proteins known to specifically bind aminoglycosides.

Although we cannot rigorously rule out the possibility that a membrane protein function is inactivated by the aminoglycosides, we favor the idea that these antibiotics exert their effects through lipid functions associated with endosome fusion. Although the IC$_{50}$ for inhibition of fusion activity (~45 μM) is consistent with the reported binding affinities of neomycin for PIP$_2$ (K$_D$ = 11–46 μM) (43), PI-PLC§1 (which binds PIP$_2$ with a K$_D$ = 210 nM (27)), does not interfere with endosome fusion suggesting that the effects of this drug do not simply reflect the specific binding and sequestration of this particular phospholipid as an essential substrate or cofactor. Neomycin also has been reported to bind other acidic lipids, including PA, PI, phosphatidylserine, and phosphatidylinositol 4-phosphate, with high affinity (K$_D$ ~ 10–250 μM) (38, 48), and therefore it is conceivable that aminoglycoside interactions with any or all of these negatively charged phospholipids promote inhibition of endosome fusion. Potential membrane alterations induced by the binding of aminoglycosides to acidic phospholipids have been widely studied because of the nephrotoxicity associated with their use as antibiotics. Not only do aminoglycosides cause a vacuolarization of apical endosomes in renal tubules (23, 24), but Giurgea-Marion et al. (25) also have shown that gentamicin prevents the delivery of horseradish peroxidase to the lysosome. As the amount of marker internalized in the latter study was not altered by gentamicin, it is very likely that this aminoglycoside blocked endosome fusion in vivo. Our in vitro experiments are entirely compatible with the idea that these antibiotics interfere with early steps in the endocytic pathway through interactions with acidic lipids.

Two distinct mechanisms have been described for the action of aminoglycoside antibiotics (reviewed in Ref. 20). The substrate depletion model proposes that by binding to specific lipids (e.g. the phosphatidylinositol); aminoglycosides can block the substrates of lipid-modifying enzymes. For example, endosome fusion appears to require a wortmannin-sensitive PI 3-kinase (49–51), and neomycin has been reported to inhibit such an activity (52). A strong argument against this model is the observed lack of effect of PI-PLC§1 on endosome fusion, as the PH domain of this factor would be expected to bind other acidic lipids in addition to PIP$_2$ (26). In contrast, the charge neutralization model suggests that by binding to acidic phospholipids, changes in local charge at the membrane bilayer interface induced by the polyaminated aminoglycosides influence the function of phospholipases (20). For example, the action of PLA$_2$ and PLB$_2$ toward a neutral substrate (PC) is strongly affected by bilayer composition and environment, such that charge neutralization as a result of aminoglycoside binding effectively lowers the activity of these enzymes (53–56). It is critical to note that a functional role for PLA$_2$ in endosome fusion has been characterized (57); therefore, this phospholipase must also be considered as a potential target of aminoglycoside inhibition. Importantly, the reversal of aminoglycoside inhibition by polyaspartic acid indicates that their effects are indeed electrostatic in nature. Such charge neutralizing effects further suggest that the lateral organization of acidic lipids within the membrane bilayer itself may play a generalized role in endocytic membrane traffic. Thus, it is very interesting to note that even in the absence of GTPγS, neomycin appears to release AP-2 from membranes but does not affect AP-1 association (17). The latter observation is completely consistent with our hypothesis that the effects of aminoglycosides are unrelated to inhibition of PLD stimulation by ARF (which appears to recruit both AP-1 and AP-2 to membranes) and furthers the argument for a key role for acidic phospholipids in endosomal membrane traffic (where AP-2 function appears to be involved). Despite the uncertainty regarding the precise mechanism of aminoglycoside action, our study rigorously demonstrates: 1) neomycin and Gentamicin are specific inhibitors of endosome fusion in vitro; 2) both antibiotics reduce the rate and extent of cell-free fusion activity, 3) the aminoglycosides block a stage of this reaction mechanism temporally related to steps inhibited by GTPγS-ARF, and 4) the mechanism of inhibition is unrelated to the PIP$_2$-dependent PLD activity regulated by ARF.

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