JOURNAL OF VIROLOGY, Nov. 1987, p. 3388–3393
0022-538X/87/113388-06$02.00/0

Vol. 61, No. 11

Evidence that Neomycin Inhibits Binding of Herpes Simplex Virus Type 1 to the Cellular Receptor

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Received 6 April 1987/Accepted 15 July 1987

The effect of neomycin, a phosphoinositide-binding aminoglycoside, on herpes simplex virus type 1 (HSV-1) infection of BHK cells was studied. We showed earlier that it specifically inhibits HSV-1 production but not HSV-2 production (Langeland et al., Biochem Biophys. Res. Commun. 141:198–203, 1986). We now show that neomycin had no effect on cellular protein synthesis, as judged by the appearance of ^{35}S-labeled polypeptides separated by polyacrylamide gel electrophoresis. Virus-induced polypeptides, however, were strongly inhibited at neomycin concentrations above 2 mM. Comparison among different aminoglycosides showed a variation in inhibition of HSV-1 production that paralleled the cationic charge of the aminoglycosides. HSV-1 receptor binding at 4°C was completely inhibited by neomycin. At 37°C both receptor binding and internalization, as measured by an indirect assay, appeared to be inhibited by more than 90%. The effect of neomycin on the infection was almost immediate upon the addition of the drug and preceded virus internalization. Possible mechanisms of the neomycin effect are discussed.

Neomycin and other aminoglycoside antibiotics can inhibit phosphoinositide-mediated events in eucaryotic cells by binding to these lipids and thereby inhibiting their hydrolysis (3, 5, 6, 15, 21). In the cochlea (8, 17, 23) and kidney (22) this has been linked to the ototoxic and nephrotoxic effects of these antibiotics. Neomycin has also been shown to inhibit phosphoinositide-mediated, Ca^{2+}-dependent secretion in mast cells (5), Ca^{2+}-dependent exocytosis in sea urchin eggs (27), inositol phosphate release from erythrocyte ghosts (6), and thrombin-stimulated growth of fibroblasts (3). In kidney brush border membranes, the cellular binding site for gentamicin was determined to be a phosphoinositide, and the different aminoglycosides were found to compete for the same binding site (20). Marche et al. (15) demonstrated that the potency of the aminoglycoside binding to phosphoinositides was related to the cationic charge of the aminoglycosides. The same order of potency has been found in the ability of the aminoglycosides to inhibit Ca^{2+}-induced hydrolysis in nerve ending membranes (26).

On the basis of the above, we wanted to investigate whether neomycin had any effect on herpes simplex virus (HSV) infection of BHK cells, which is accompanied by altered turnover of phosphoinositides (10). In a previous report (11), we showed that neomycin selectively inhibited HSV type 1 (HSV-1) infection, whereas HSV-2 infection was unaffected. Importantly, neomycin had to be present at the time of infection to exert maximal effect, whereas the addition of the antibiotic 1 h postinfection had no effect. These results suggested that neomycin might interfere at the receptor level. HSV-1 and HSV-2 have separate cellular receptors (1, 25). The HSV-1 receptor is probably a glycoprotein (16, 29); however, no phospholipid involvement with the HSV-1 receptor has been reported. In other systems we know that phosphoinositides may be involved in hydrophobic "anchoring" of plasma membrane glycoproteins (12, 13); covalent binding of phosphoinositides to myelin basic protein has also been reported (4, 28).

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The results reported in this communication indicate that neomycin affects virus receptor binding. We also show that the order of potency of different aminoglycosides correlates with their cationic charge. The possibility that either the virus or the cellular receptor, or both, depends on phosphoinositides for normal function is discussed.

MATERIALS AND METHODS

Materials. Eagle minimum essential medium (EMEM) and newborn calf serum were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland). Neomycin, gentamicin, and streptomycin (sulfate form) were from Sigma Chemical Co. (St. Louis, Mo.). Cell culture dishes were from Nunc (Roskilde, Denmark). Fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin (DAKO F232) was obtained from DAKOPATTS (Copenhagen, Denmark). [^{35}S]methionine (SJ 204; 1270 Ci/mmol) was from Amersham International (Amersham, Buckinghamshire, England).

Cell culture. BHK21 clone 13 cells (14) were grown to confluence in 32-mm culture dishes in EMEM supplemented with 10% newborn calf serum.

Virus stocks and antibodies. The virus strains used were HSV-1 strain 17 Syn$^+$ (2) and HSV-2 strain HG52 (24). Mouse serum containing polyclonal antibodies directed against HSV thymidine kinase was a generous gift from Kenneth Powell, Wellcome Research Laboratories, England.

Indirect immunofluorescence. Cells were grown on glass cover slips in culture dishes. Neomycin was added as described below, and infection was carried out at a multiplicity of 5 PFU per cell. The cells were fixed in methanol at −20°C 6 h postinfection. Further procedure was essentially as described by Addison et al. (1).

Radioisotopic labeling of proteins. Cells were either infected at a multiplicity of 10 PFU per cell or mock infected. Incubations were carried out in the absence or presence of neomycin (in increasing amounts). From 1 to 6 h postinfection, cellular proteins were labeled with 50 μCi of [^{35}S]methionine per ml. Cells were then scraped off the culture

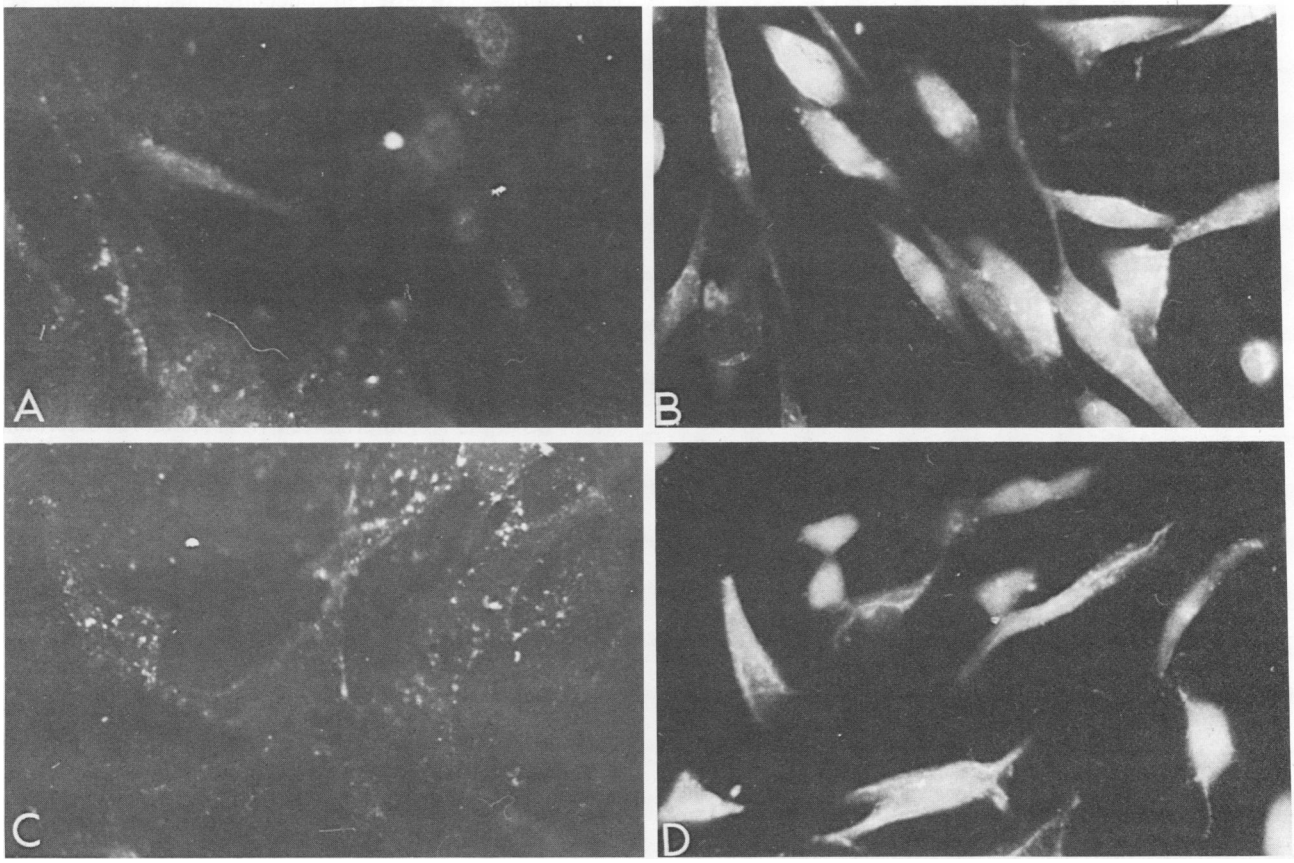

FIG. 1. Effect of neomycin on the immunofluorescent appearance of viral thymidine kinase in BHK cells infected with HSV-1. Cells were either treated with 5 mM neomycin during infection (panels C and D) or given no neomycin treatment (panels A and B). (A) Mock-infected cells; (B) infection with 5 PFU per cell; (C) neomycin added 10 min before infection; (D) neomycin added 1 h postinfection.

dishes and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described below.

Polyacrylamide gel electrophoresis. Cells were harvested, and radioactively labeled polypeptides were analyzed on a 5 to 12.5% gradient gel cross-linked with *N*,*N*′-methylenebisacrylamide as described previously (7). The gels were exposed to Kodak XAR-5 films (Eastman Kodak Co., Rochester, N.Y.) for 2 days.

Receptor-binding studies and measurement of virus growth in the presence of aminoglycosides. The individual aminoglycosides were dissolved in complete EMEM, the pH of the solutions was adjusted to 7.40, and the drugs were added to the cells at the indicated times and concentrations. The cells were infected with 30 to 50 PFU per dish. In some experiments (Table 1), infection was performed at 4°C and further incubations were carried out for 2 h at this temperature. Neomycin was present from infection and throughout this period. Before further incubation in complete EMEM at 37°C the cells were washed with phosphate-buffered saline (PBS) at pH 7.4 or pH 3.0 as indicated. Plaque formation was recorded 48 h postinfection. Internalized virus was defined as the plaque yield of the cells washed with PBS at pH 3.0; washing with neutral PBS preserved both internalized and receptor-bound virus, and this plaque yield was termed receptor-bound virus.

RESULTS

Effect of neomycin on the synthesis of virus-specific proteins. The effect of neomycin treatment at various times before or after infection on virus-specific protein synthesis was studied by immunofluorescence and polyacrylamide gel electrophoresis.

When 5 mM neomycin was added 10 min before infection, the amount of viral thymidine kinase formed 6 h later was not significantly greater than the background determined in uninfected cells (Fig. 1A and C). Drug addition 1 h postinfection allowed virus-specific thymidine kinase to accumulate at the same rate as in cells infected in the absence of neomycin (Fig. 1B and D).

Gel electrophoresis of polypeptides labeled with [^{35}S]methionine is shown in Fig. 2. Neomycin (5 mM) had little effect on the total amount of radioactivity incorporated into cellular proteins (less than 15% reduction in trichloroacetic acid precipitate; results not shown) or on the relative intensity of the individual bands (lanes a and b). The pattern of viral polypeptides shown in lane c changed gradually when the concentration of neomycin was increased from 2 mM and upward (lanes f through h). Virus-specific proteins were undetectable at a drug concentration of 5 mM. As expected from previous experiments (11), concentrations below 2 mM had no effect.

Comparison among aminoglycoside antibiotics. The effect of different aminoglycoside antibiotics on HSV-1 plaque formation was compared (Fig. 3). Each antibiotic was added 10 min before infection and kept in the medium until 1 h postinfection, at which time the medium was removed and replaced with fresh EMEM and the cells were further incubated for plaque assay. The various antibiotics were in

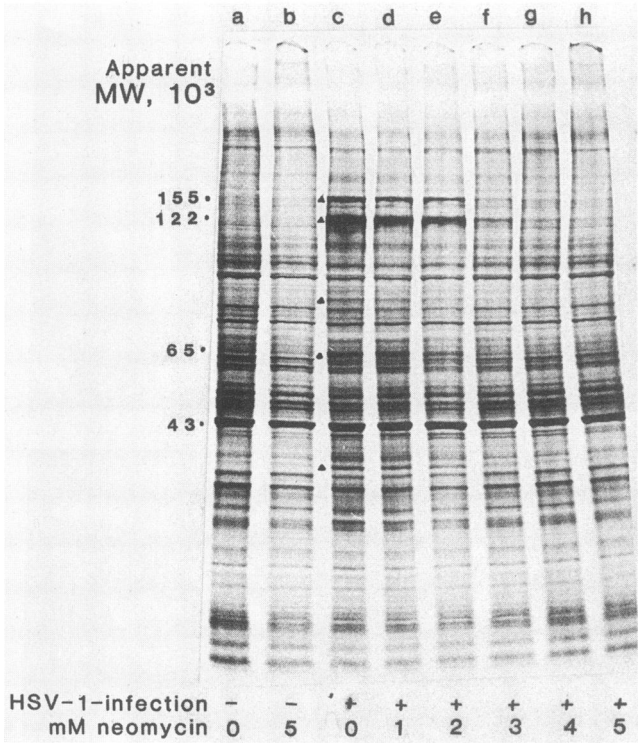

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mock-infected and HSV-1-infected BHK cells treated with neomycin at various concentrations. Cells were either mock infected (lanes a and b) or infected (lanes c to h) for 6 h. Labeling with [^{35}S]methionine was performed as described in Materials and Methods. When present, neomycin was added 10 min before infection at the concentrations shown below each lane. Some prominent virus polypeptides are indicated (▲).

the form of sulfate salts; sulfate at corresponding concentrations had no effect on plaque formation (data not shown). Figure 3 shows that there was good correlation between the effects of the individual drugs and their cationic charges, which can be ranked in the following order: neomycin, 6+; gentamicin, 5+; streptomycin, 3+. We also tested whether the present data were merely a result of the presence of charged amino groups. Spermine, poly-L-lysine, and glucosamine were tested in concentrations corresponding to the above amino group charges (data not shown). Spermine and poly-L-lysine were toxic at such concentrations, whereas glucosamine up to 40 mM was not toxic and had no effect on plaque formation. Spermine was not toxic below 100 μM and did not have any effect on plaque formation at these concentrations. However, poly-L-lysine (M_r, 3,700) at a nontoxic concentration (10 μM) showed an effect on plaque formation that was very similar to that of neomycin at millimolar concentrations. Thus, the amino group charge alone was not the cause of our observations.

Receptor-binding studies. In the following experiments the neomycin concentration was 5 mM. The cells were exposed to neomycin from 10 min before infection and until PBS washing. Receptor-bound plus internalized virus was measured as virus which was not removed by repeated washing with PBS at pH 7.4. Internalized virus alone was determined by washing the cells with PBS at pH 3.0; this treatment removed receptor-bound virus, whereas internalized virus was protected (9, 19). In the first set of experiments (Fig. 4), PBS was added at the indicated times to remove unbound pH 7.4; Fig. 4A) or not internalized (pH 3.0; Fig. 4B) virus. One hundred percent plaque formation was defined as the number of plaques formed without PBS or neomycin treatment. Internalization was complete after approximately 60 min in untreated cells (data not shown), whereas in the neomycin-treated cells internalization was reduced to 1/20 of the value determined in untreated cells (Fig. 4B). Receptor binding seemed to be blocked by neomycin treatment of the cells by approximately the same factor (Fig. 4A).

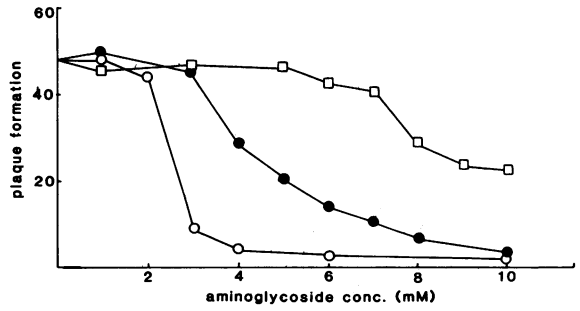

FIG. 3. Comparison of the effects of various aminoglycosides on HSV-1 infection. Cells were incubated with the aminoglycosides at the concentrations given on the abscissa from 10 min preinfection and until medium was removed 1 h postinfection. Fresh medium was added, and the dishes were incubated further for plaque assay. Infection was performed at 50 PFU per dish. Symbols: (○) neomycin, (●) gentamicin, (□) streptomycin.

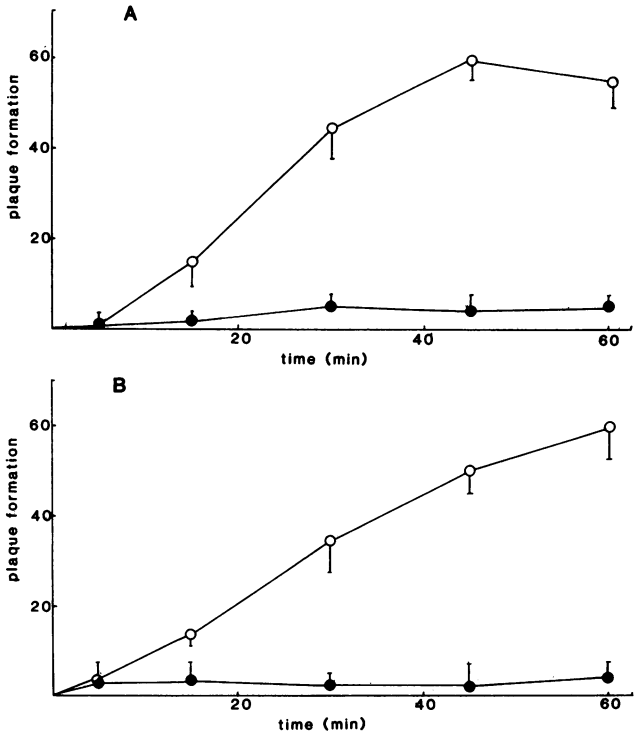

FIG. 4. Effect of neomycin on receptor binding and internalization of HSV-1. Cells were treated with 5 mM neomycin (●) or not treated (○). Cells were washed either with PBS at pH 7.4 (A) or pH 3.0 (B) at the indicated times. Plaque formation was recorded 48 h postinfection. Data are given as means ± standard deviations of three separately incubated dishes.

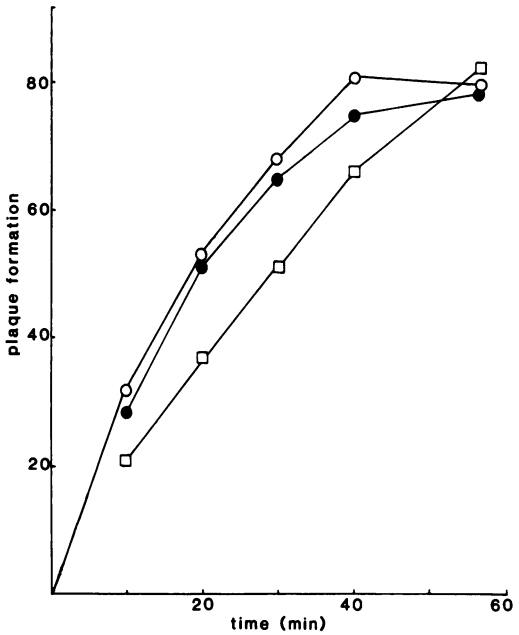

FIG. 5. Time courses of HSV-1 receptor binding and internalization compared with effect of neomycin addition at various times postinfection. Receptor-bound virus at various times postinfection was determined by washing with PBS at pH 7.4 at the indicated times (○). Internalized virus at various times was determined by washing with PBS at pH 3.0 at the indicated times (□). Neomycin (5 mM) was added at the times indicated (●); these cells were not PBS treated, infection was continued until 90 min postinfection, the medium was changed, and then the cells were further incubated for the plaque assay.

We also tested whether neomycin had any irreversible effect on the virus particle or the cells that made infection impossible. The virus and the cells were incubated separately at 37°C with increasing concentrations of neomycin under conditions otherwise exactly as described in the legend to Fig. 3. After 1 h of incubation, the neomycin-treated virus had, when diluted below inhibiting neomycin concentrations, the same infectivity as the untreated virus. Likewise, neomycin treatment of the cells did not affect subsequent viral infection (data not shown). Thus, neomycin had no irreversible effect on viral or cellular components necessary for infection.

Table 1 shows the results of experiments comparing receptor binding of HSV-1 and HSV-2 in response to neomycin treatment. Infection was performed at 4°C, at which receptor binding can take place but internalization is not permitted (9). The data show that more than 90% of HSV-1 was removed by washing with PBS at pH 3.0, whereas only 50% of HSV-2 was removed. Neomycin inhibited HSV-1 receptor binding, whereas HSV-2 receptor binding and internalization were unaffected. This result correlated well with our earlier observation that HSV-2 infection was altogether unaffected by neomycin treatment (11).

Figure 5 shows results from experiments in which the time courses of receptor binding and internalization of HSV-1 were compared. The effect of neomycin addition at various times postinfection is also shown. The data for receptor binding were obtained by washing with PBS at pH 7.4, and the internalization data were obtained by washing with PBS at pH 3.0, at the times indicated. Neomycin treatment was performed from the indicated times and until 90 min postinfection, at which time the cells were washed and incubated further for plaque assay. Thus, the neomycin-treated cells did not receive any PBS treatment during the 60-min time period shown in Fig. 5. The time lag from receptor binding until the virus was internalized averaged approximately 8 to 10 min (Fig. 5). The amount of internalized virus equaled that of receptor-bound virus at 60 min postinfection, indicating that all of the infectious virus particles were internalized by this time. The neomycin effect was very fast; the observed effect was only approximately 1 min delayed compared with receptor binding. Even this delay may be artifactual, taking into consideration that neomycin treatment was not completely effective (5 to 10% of virus entry was not inhibited [Fig. 3]). In addition, as noted above, the experimental conditions of the neomycin- and receptor-binding curves were not identical; this by itself yields some experimental uncertainty. It still seems convincing from the data in Fig. 5 that neomycin blocked HSV-1 infection at a time very close to the receptor-binding time and that this inhibitory effect was almost immediate upon the addition of the drug.

DISCUSSION

Our previous finding that neomycin blocked the production of HSV-1 virus in BHK cells by more than 90% (11) led us to investigate further the site of action of this drug. Neomycin binds strongly to phosphoinositides and is a highly charged, hydrophilic molecule which is not likely to penetrate biological membranes; furthermore, polyphosphoinositides are mainly located in the plasma membrane. These facts and our finding that neomycin must be present at the time of infection to exert full inhibitory effect prompted us to investigate whether the target for neomycin was at the plasma membrane level, i.e., at very early stages of HSV-1 infection.

We first demonstrated by immunofluorescence that neomycin almost completely blocked HSV-1 infection when present at the time of infection, whereas the addition of the drug 1 h postinfection led to a seemingly normal infection of the cells (Fig. 1). Thus, when the first steps of infection have occurred, the presence of neomycin does not interfere with the further, normal course of infection.

Neomycin did not have any major effect on the host cellular protein synthesis (Fig. 2, lanes a and b). Thus, the results presented in Fig. 1 and 2 suggest that neomycin did not block multiplication of HSV-1 by a general effect on translation, but inhibited a step (or steps) before the synthesis of virus-encoded proteins. The ability of neomycin to bind to the smaller ribosomal subunit and thus inhibit bacterial growth (18) seems to be limited to procaryotic cells.

TABLE 1. Receptor binding and internalization of HSV-1 and HSV-2 at 4°C in the absence or presence of neomycin[a]

Infection		% Plaque formation after washing with PBS at:	
Virus	Neomycin (mM)	pH 7.4	pH 3.0
HSV-1	0	100	7.2
HSV-1	5	6.5	6.1
HSV-2	0	100	49.2
HSV-2	5	104.3	47.2

[a] Data are given as percentages. One hundred percent plaque formation was defined as the number of plaques obtained without neomycin treatment and with washing with PBS at pH 7.4. Results are means of three separate experiments. The standard deviations were within the range of ±10%.

Research on aminoglycoside effects in eucaryotic cells has mainly been directed toward their toxicity, which is a clinical problem. The toxic potency is dependent upon their cationic charge (15); so is their capacity to bind to polyphosphoinositides, but this is not related to their antibacterial effect. Our finding that the aminoglycosides have a decreasing antiviral potency in the order neomycin, gentamicin, streptomycin (Fig. 3) correlates well with their cationic charges. It is thus possible that an interaction between these drugs and the highly negatively charged phosphoinositides causes the observed effect on HSV-1 infection.

Evidence that the effect of neomycin on HSV-1 infection was on binding to the receptor rather than on internalization of the virus is presented in Fig. 4 and 5 in Table 1. Virus still bound to the receptors were dissociated from the cells by washing at low pH. This treatment did not detectably affect multiplication of intracellular virus since the final yield was the same as after washing at pH 7.4 (Fig. 5). We also tested whether it affected the virus particle itself. Preincubation of virus in PBS at pH 3.0 for 10 min did not affect subsequent infection (data not shown). Neomycin inhibited almost completely the internalization of HSV-1 (Fig. 4). When virus was allowed to bind to the receptors but inhibited from translocation into the cells (Table 1), approximately 95% of HSV-1 that had attached (pH 7.4 wash) came off at pH 3.0. This reversible binding of HSV-1 was almost completely blocked by 5 mM neomycin. The incomplete effect of washing with PBS at pH 3.0 could be explained if the acid wash as a technique were only 95% effective. Alternatively, the virus might enter the cell through two different mechanisms, one receptor mediated and one unspecific.

If either receptor proteins on the cell surface or receptor-binding proteins in the virus envelope were anchored in the phospholipid membrane through an inositol phospholipid, this would explain our findings. Neomycin bound to the phospholipid part of this complex could then induce steric changes which would inhibit HSV-1 binding. Since the order of potency of inhibition correlated with the cationic charge of the drug, it seemed likely that the binding between drug and phospholipid is ionic.

We tried to measure the effect of neomycin on the metabolism of the cellular phospholipids directly. The relative increase in phosphatidylinositol 4,5-bisphosphate after HSV-1 infection which we reported earlier (10) was abolished by neomycin treatment (data not shown). However, this can not readily be explained as binding of neomycin to the lipid, since it is more likely a result of an altogether abolished infection, as demonstrated in this publication. In addition, since the observed neomycin effect was on receptor binding, and most of the phosphoinositides are probably located at the inner leaflet of the plasma membrane and not readily accessible for neomycin, an effect of neomycin on the small portion of phosphoinositides present in the outer leaflet of the cell membrane would not be anticipated to have any major effect on the total mass of inositol lipids.

In conclusion, we have in this communication given evidence that neomycin specifically inhibited HSV-1 receptor binding. Cellular protein synthesis and virus-induced protein synthesis seemed unaffected when neomycin was present at concentrations below 2 mM. At higher concentrations the virus-induced protein synthesis was reduced, suggesting the actual infection to be much lower than what was expected based on the added multiplicity of virus. Our data suggest a role for the phosphoinositides in the HSV-1 virus-receptor interaction. Further studies are required to confirm this possibility.

ACKNOWLEDGMENTS

Sigrid Øvernes provided excellent technical assistance.

The financial support from The Norwegian Society for Fighting Cancer is acknowledged.

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