

Inhibition of Poliovirus RNA Synthesis by Brefeldin A

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Received 7 October 1991/Accepted 20 December 1991

Brefeldin A (BFA), a fungal metabolite that blocks transport of newly synthesized proteins from the endoplasmic reticulum, was found to inhibit poliovirus replication 10⁵- to 10⁶-fold. BFA does not inhibit entry of poliovirus into the cell or translation of viral RNA. Poliovirus RNA synthesis, however, is completely inhibited by BFA. A specific class of membranous vesicles, with which the poliovirus replication complex is physically associated, is known to proliferate in poliovirus-infected cells. BFA may inhibit poliovirus replication by preventing the formation of these vesicles.

Poliovirus is a nonenveloped, icosahedral RNA virus. Poliovirus genome replication, protein synthesis, and virion formation occur in the cytoplasm of infected primate cells. Poliovirus enters the cell via its receptor on the plasma membrane, a member of the immunoglobulin superfamily of proteins (29). Following entry, the 7,500-nucleotide positive-sense poliovirus genome directs the synthesis of a 220-kDa polyprotein. The polyprotein is subsequently processed into smaller polypeptides, including at least five polypeptides (2B, 2C, 3AB, 3CD, and 3D, the viral RNA-dependent RNA polymerase) involved in the replication of viral RNA (33).

During poliovirus infection, drastic alterations in cellular morphology and metabolism occur. These changes, collectively known as the cytopathic effect, are the results of the inhibition of host cell protein synthesis (17, 39), the reorganization of the cytoskeleton (24, 44), and the proliferation of membranous vesicles (3, 4, 9, 15, 33). The inhibition of host cell protein synthesis is mediated by viral protein 2A (2). Even the small amount of 2A translated from the input genomic RNAs at high multiplicities of infection (MOIs) leads to the cleavage of the host cell's mRNA cap-binding protein p220 and the subsequent inactivation of the cap-binding complex required for translation of almost all cellular mRNAs (39). Poliovirus RNA is translated by a cap-independent mechanism (31, 46). Inhibition of host cell translation presumably facilitates poliovirus infection by freeing ribosomes from cellular mRNAs and thus increasing the rate of accumulation of poliovirus proteins (2, 39).

The functions of other changes in the host cell during poliovirus infection are beginning to be better understood as well. For example, the poliovirus replication complex is associated with the membranous vesicles that proliferate during infection. The interaction between the replication complex and these vesicles is thought to be mediated by the viral 2C and 3AB polypeptides (3, 32). Concomitant with the formation of these vesicles, there is an increase in phosphatidylcholine synthesis (9). An inhibitor of lipid synthesis, cerulenin, inhibits poliovirus RNA synthesis, suggesting that the formation of these vesicles is at least in part a result of de novo lipid synthesis and that these vesicles are required for RNA replication (18). The origin of the vesicles associated with the poliovirus replication complex and the mechanism of their formation are not known. Electron microscopic observations suggest that at least some of the poliovirus-

induced vesicles are formed by budding from the endoplasmic reticulum (ER) (9, 15).

The fungal metabolite brefeldin A (BFA) has been used to study membrane traffic in eukaryotic cells (16, 25, 26, 29, 30, 40). BFA blocks protein transport from the ER to the Golgi apparatus (16, 25, 26, 29). There appears to be an ongoing, two-way traffic between the ER and the Golgi apparatus. BFA, by inhibiting the ER-to-Golgi path, leads to the redistribution of Golgi membranes and proteins into the ER (16, 25, 29). BFA also inhibits the formation of non-clathrin-coated vesicles from the Golgi apparatus *in vitro* (30). BFA does not affect other cellular processes involving membranes such as endocytosis, endosome acidification, or lysosome function (29). Since BFA is thought to inhibit the formation of specific classes of vesicles (30), we reasoned BFA might also block the proliferation of vesicles in poliovirus-infected cells. In fact, BFA is one of the most potent inhibitors of poliovirus replication yet described, specifically blocking poliovirus RNA replication.

MATERIALS AND METHODS

Cell culture and drug treatment. HeLa cells were grown in spinner culture in minimal essential medium (MEM) (Sigma) supplemented with 7% horse serum (GIBCO), 100 U of penicillin G per ml, and 100 µg of streptomycin sulfate per ml (MEM/complete). For growth on plates, cells were kept in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% calf serum (GIBCO), and penicillin G and streptomycin as described above (DMEM/complete). BFA (Epicentre Technologies) was stored as a 5-mg/ml solution in ethanol, nocodazole (Sigma) was stored as a 10-mg/ml solution in dimethyl sulfoxide, guanidine-HCl was stored as a 100 mM solution in phosphate-buffered saline (PBS), and actinomycin D (Sigma) was stored as a 5-µg/ml solution in water.

Poliovirus infections and RNA transfections. HeLa cells were washed once in PBS supplemented with 0.01% CaCl₂ and 0.01% MgCl₂ (PBS+). Mahoney type I poliovirus adsorption was in PBS+ for 30 min at 37°C unless otherwise indicated. Following infection, cells were washed with PBS+ to remove unabsorbed virus and then grown in MEM/complete (for spinner cultures) or DMEM/complete (for plates). At the indicated times postinfection, samples were harvested, cytoplasmic extracts were prepared and plaque assays were performed as described previously (22).

For poliovirus RNA synthesis measurements, HeLa cells were infected with poliovirus and grown in the presence or

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absence of 2 μg of BFA per ml and in the presence of 2 μg of actinomycin D (Sigma) per ml to inhibit cellular transcription. [^3H]uridine was added either immediately following infection or at 2 or 3 h postinfection. For cold uridine chases, a 0.1 M solution of uridine was added to the medium at the appropriate time to a final concentration of 10 mM. Treatments of uninfected cells with BFA and actinomycin D were identical to similar treatments of infected cells. Cells were harvested and trichloroacetic acid precipitations were performed as described previously (22).

Direct transfection of poliovirus RNA was accomplished using the Baekon 2000 electric field-mediated transfer system (Baekon, Inc., Saratoga, Calif.). A total of 8×10^7 HeLa cells from a spinner culture were collected by centrifugation at $800 \times g$ for 5 min, washed twice with PBS+, and resuspended in 200 μl of PBS+. Poliovirion RNA (0.5 μg) was added to the cells and an electric field in noncontact mode (7,500 V, 2^8 pulses per cycle, 5 cycles, 160-ms pulse width, 3.2-s burst time) was immediately applied. Samples (50 μl) of electroporated cells were transferred to 30-ml portions of MEM/complete in the absence or presence of 2 μg of BFA per ml. Incubation with stirring was continued at 37°C; at the indicated times, 3-ml samples were harvested, the cells were collected, cytoplasmic extracts were prepared, and plaque assays were performed as described previously (22).

Adenovirus infections and measurements of adenovirus DNA synthesis. Infections with phenotypically wild-type *dl309* adenovirus serotype 5 (a gift from J. Schaak, University of Colorado Health Sciences Center) were performed at an MOI of 5 as described by Jones and Shenk (21). Petri dishes containing 5×10^6 HeLa cells each were infected with adenovirus and incubated with DMEM/complete supplemented with 10% fetal calf serum (Sigma) for 48 h. Treatments with 2 μg of BFA per ml were for 12-h intervals during the 48-h time period; no cells were exposed to BFA for more than one 12-h interval. For each set of infections, 5×10^6 cells were harvested at 0, 12, 24, 36, and 48 h postinfection. Virus yield was determined for the 48-h time points by plaque assay as described by Schaak et al. (37).

To measure the amount of adenovirus DNA synthesis during infection, whole-cell extracts from 5×10^6 cells were prepared and phenol extracted, and the nucleic acids were collected by ethanol precipitation (37). Dot blot analysis was performed using a Lucite manifold (Schleicher and Schuell) and a ^{32}P -labeled adenovirus DNA probe as described previously (37).

Immunofluorescence microscopy. HeLa cells were plated onto glass coverslips and grown for at least 36 h in DMEM/complete prior to use. Cells were infected with poliovirus at an MOI of 10 as described above and then grown in DMEM/complete in the presence or absence of BFA at concentrations of 1 to 5 $\mu\text{g}/\text{ml}$. At 5 h postinfection, cells were washed once with PBS+, fixed in 100% methanol at -20°C for 10 min, and stained with a rabbit polyclonal anti-virion antiserum (a gift of P. Sarnow, University of Colorado Health Sciences Center). Bound antibody was visualized using a goat anti-rabbit immunoglobulin G antibody conjugated to rhodamine (Boehringer Mannheim).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis. Infected cells grown in the presence of 2 μg of BFA per ml, 1 mM guanidine, or no antiviral compounds were labeled with [^{35}S]methionine at 50 $\mu\text{Ci}/\text{ml}$ in serum-free medium for 20-min intervals at various times postinfection. After the cells were labeled, 2×10^6 to 2.5×10^6 cells were harvested

at each time point and dissolved in 0.5 ml of lysis buffer (50 mM Tris HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) as described above. For one-dimensional gels, a 20- μl aliquot of each lysate was mixed with 20 μl of $2\times$ Laemmli buffer (23), boiled for 5 min, and loaded onto a 15% acrylamide gel. These gels were blotted onto a supported nitrocellulose membrane (MSI) or dried onto 3MM paper and exposed to Kodak XAR5 film at -70°C to visualize total protein. For two-dimensional gels, cells were recovered by centrifugation and total protein was solubilized in 9 M urea; first and second dimensions were run as described previously (1). Two-dimensional gels were stained with Coomassie brilliant blue, destained, and enhanced for autoradiography following manufacturer's instructions (Auto-fluor; National Diagnostics).

Lipid synthesis measurements. Synthesis of phosphatidylcholine and its incorporation into lipid was measured by monitoring the uptake of [^3H]choline into the organic-lipid-containing phase of cell lysates. Synthesis of glycerol lipids was monitored by measurements of uptake of [^{14}C]glycerol. Uninfected and infected cells were grown as monolayer cultures in the presence or absence of 2 μg of BFA per ml. Immediately following infection, both [^3H]choline and [^{14}C]glycerol were added to the growth medium (DMEM/complete with 2% calf serum instead of 10%) at concentrations of 1 $\mu\text{Ci}/\text{ml}$ each. At various times postinfection, samples of 2.2×10^6 cells each were washed twice with 0.15% NaCl and lysed with 0.2% SDS in saline. Total lipids were extracted from an aliquot of each lysate with methanol and CHCl_3 by the method of Bligh and Dyer (7). The resulting organic-lipid-containing phase was dried under a stream of nitrogen gas and resuspended in scintillation cocktail (Aquasol), and the amount of each isotope was determined by scintillation counting (Beckman).

RESULTS

BFA inhibits poliovirus replication. To test the effects of BFA on the production of poliovirions, cells were treated with various concentrations of BFA immediately following poliovirus infection. Control cells were exposed to an appropriate amount of ethanol, the solvent used to dissolve BFA. The production of poliovirus capsid proteins and virions was monitored by immunofluorescence microscopy. At BFA concentrations of 2 and 5 $\mu\text{g}/\text{ml}$, virion staining (Fig. 1a) was completely inhibited (Fig. 1c and d). At 1 μg of BFA per ml, the number of anti-poliovirus staining cells was greatly reduced (Fig. 1b); however, those cells that were stained by the antibody were stained as strongly as control, i.e., poliovirus-infected cells (Fig. 1a and b). This experiment provided the first evidence that BFA inhibits poliovirus infection. We chose to use a BFA concentration of 2 $\mu\text{g}/\text{ml}$ for subsequent experiments, since it produced a complete inhibition of virion production as assayed by immunofluorescence microscopy and eliminated the variability of inhibition seen at 1 μg of BFA per ml. This concentration of BFA is comparable to those used by others to document the specific effects of BFA on protein secretion; these concentrations have ranged from 1 $\mu\text{g}/\text{ml}$ (29) to 10 $\mu\text{g}/\text{ml}$ (26).

To characterize the effect of BFA on poliovirus infection further, cells were infected with poliovirus at an MOI of 0.25 and incubated in the presence or absence of 2 μg of BFA per ml. The yield of infectious poliovirus from equal numbers of cells was determined from 0 to 8 h postinfection. Figure 2a shows the observed reduction in poliovirus titer, a decrease of 5 to 6 orders of magnitude in the presence of BFA.

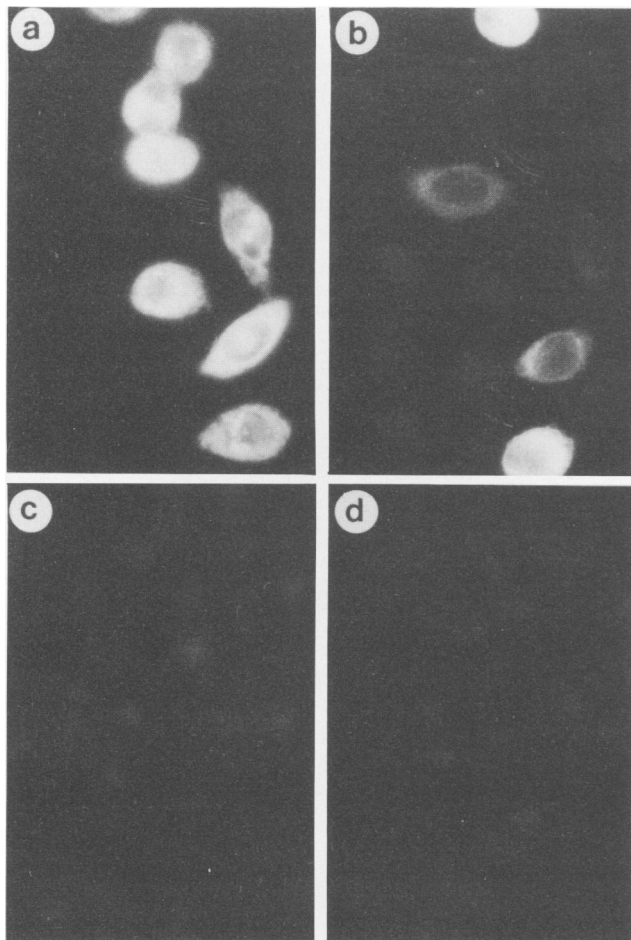


FIG. 1. Dose dependence of inhibition of poliovirus infection by BFA. HeLa cells on glass coverslips were fixed in 100% methanol 5 h after infection with poliovirus in the presence of various amounts of BFA. The cells were stained with an anti-poliovirion rabbit polyclonal antibody obtained from P. Sarnow (University of Colorado Health Sciences Center, Denver). Cells pictured were exposed to 0 (a), 1 (b), 2 (c), or 5 (d) μg of BFA per ml.

A BFA-sensitive step occurs after the entry of poliovirus into cells. To determine the step(s) in the infectious cycle at which BFA affects poliovirus growth, we examined the effects of BFA on cell entry, protein synthesis, and RNA synthesis individually. The first step in poliovirus infection is virion binding to the poliovirus receptor (28) and release of the viral mRNA into the cell. To bypass the cell entry and RNA release steps, poliovirus infection was initiated by electroporation of poliovirus RNA into HeLa cells, and the transfected cells were grown in the presence or absence of BFA. Duplicate transfections in the absence of BFA (Fig. 2b) showed viral yields greater than 10^6 PFU/ml in the resulting cytoplasmic extracts. As with virion-infected cells, BFA completely inhibited virus production in RNA-transfected cells. The virus yields in duplicate transfections incubated in the presence of BFA were lower than 5 PFU/ml at every time point examined, a reduction in titer of 6 orders of magnitude (Fig. 2b).

That this inhibition was seen even when the infection was initiated by RNA transfection is evidence that BFA affects a step in the poliovirus infectious cycle which follows the

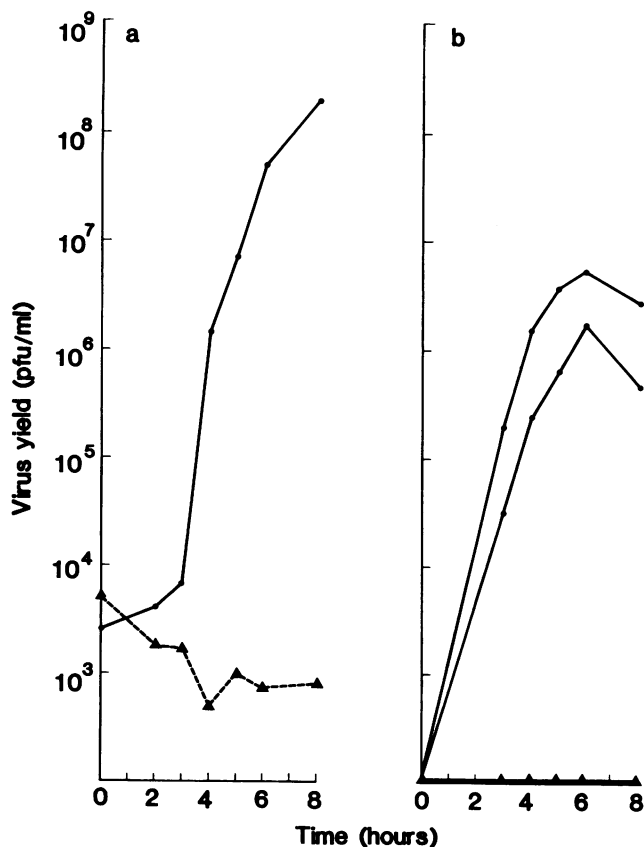


FIG. 2. Inhibition of poliovirus infection and poliovirus RNA transfection by BFA. Growth curves of poliovirus are shown for infections initiated by viral infection or by RNA transfection. (a) Time course of poliovirus infection in the absence (●) and presence (▲) of 2 μg of BFA per ml; (b) growth curves following duplicate RNA transfections in the absence (●) and presence (▲) of 2 μg of BFA per ml.

binding of the virion to its receptor and the subsequent release of the viral RNA into the cytoplasm.

BFA does not inhibit poliovirus translation. The next step leading to a productive poliovirus infection is the translation of the poliovirus genome. A sensitive assay for translation of poliovirus proteins early in infection is to monitor the inhibition of host protein synthesis. Translation of only the input RNA molecules can be followed by performing an infection at a high MOI in the presence of guanidine hydrochloride, an inhibitor of poliovirus RNA synthesis (8). Figure 3a shows the labeled proteins synthesized following infection by poliovirus in the presence (lanes 11 to 14) and absence (lanes 1 to 4) of 1 mM guanidine. Cells were labeled with [^{35}S]methionine for 20 min prior to harvest, and equal numbers of cells were harvested at 2, 3, 4 and 5 h postinfection. During poliovirus infection in the absence of the RNA replication inhibitor, both the accumulation of labeled poliovirus proteins and the inhibition of host cell protein synthesis (17, 39) were seen (Fig. 3a, lanes 1 to 4). For poliovirus infections performed in the presence of guanidine, viral proteins did not accumulate in sufficient quantities to be visualized directly on the autoradiograph shown in Fig. 3a. However, there are many fewer labeled proteins in the extract made from cells harvested at 5 h after infection in the presence of guanidine (lane 14) than are present in the

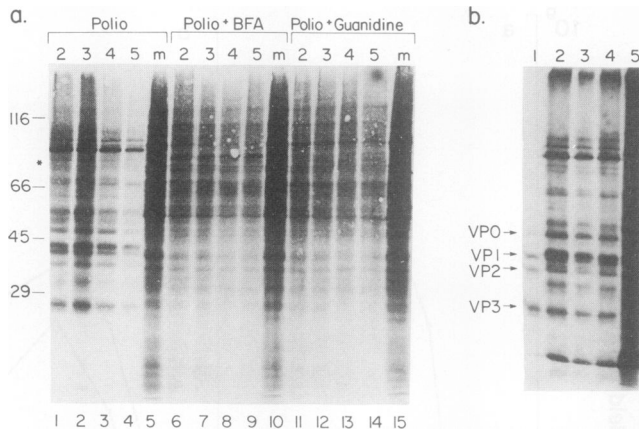


FIG. 3. Effect of BFA on translation in poliovirus-infected cells. (a) HeLa cells were infected with poliovirus (polio) and grown in the presence of no inhibitors (lanes 1 to 4), 2 μg of BFA per ml (lanes 6 to 9), or 1 mM guanidine HCl (lanes 11 to 14). Cells were labeled with [³⁵S]methionine for 20 min prior to harvest. The duration of infection in hours is marked above each lane; "m" denotes mock-infected cell extracts prepared at 5 h postinfection. Molecular sizes of marker proteins in kilodaltons are shown at the left. (b) Proteins synthesized in uninfected HeLa cells (lane 5) and in poliovirus-infected HeLa cells treated at 3 h postinfection with no inhibitors (lane 2), 2 μg of BFA per ml (lane 3), or 1 mM guanidine HCl (lane 4) are shown. Labeled poliovirus proteins VP0, VP1, VP2, and VP3 are shown (lane 1) for reference.

mock-infected cell extract (lane 15), showing that host cell translation was substantially inhibited in the presence of guanidine. Guanidine alone does not inhibit cellular translation (8). Thus, sufficient levels of poliovirus proteins, protein 2A in particular, were translated in the presence of guanidine to accomplish the inhibition of host protein synthesis.

The translation products that accumulated in the presence of 2 μg of BFA per ml are shown in lanes 6 to 9 of Fig. 3a. The time course of [³⁵S]methionine incorporation was nearly identical to that observed in guanidine-treated cells, showing that the input poliovirus RNA could be translated in the presence of BFA. Thus, translation of the poliovirus proteins early in the infectious cycle is not affected by the presence of BFA.

To determine whether translation of poliovirus RNA later in infection was affected by BFA, the inhibitor was added to the medium of HeLa cells 3 h postinfection with poliovirus. Figure 3b shows the accumulation of proteins labeled with [³⁵S]methionine during a 20-min labeling interval. This interval followed 10 min after the addition of no inhibitor (lane 2), BFA (lane 3), or guanidine (lane 4); 10 min is sufficient time for BFA to take effect (16, 26). It is apparent that viral translation late in infection, as well as early, is unaffected by the addition of BFA.

One significant difference between the ³⁵S-labeled proteins that accumulated in guanidine-treated cells and those in BFA-treated cells was an increased amount of an approximately 80-kDa protein in the infected cells treated with BFA (Fig. 3a). This 80-kDa protein comigrated with a labeled species derived from mock-infected, BFA-treated cells in one-dimensional gels (data not shown) and thus is likely to be a host protein. Sarnow (36) has shown that a host protein of similar electrophoretic mobility, glucose-regulated protein 78/immunoglobulin heavy-chain binding protein (grp78/BiP), continues to be translated by an internal ribosome-binding

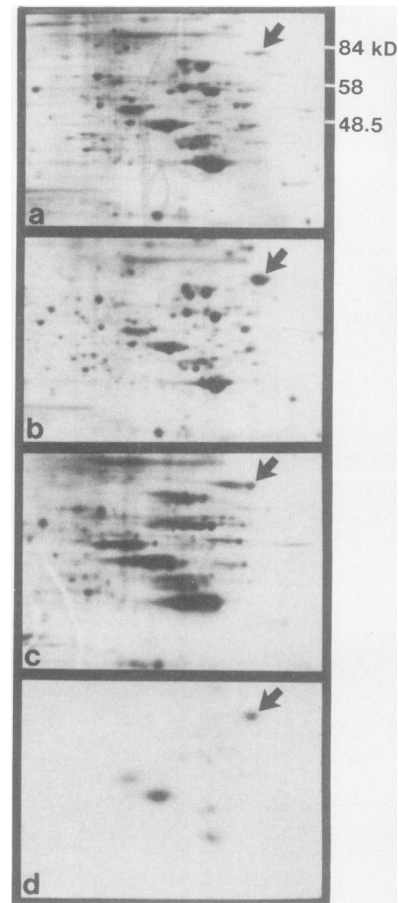


FIG. 4. BFA induces the translation of an approximately 80-kDa glucose-regulated protein. To determine whether the 80-kDa protein found to be preferentially labeled in poliovirus-infected, BFA-treated cells was a glucose-regulated protein, HeLa cells were grown in the presence (a) or absence (b) of glucose for 20 h and labeled with [³⁵S]methionine for 3.75 h at 37°C. Total cellular protein was analyzed by two-dimensional isoelectric focusing/SDS-PAGE and by autoradiography. In the absence of glucose, the translation of an approximately 80-kDa protein was greatly increased (arrow) over its level in control cells. (c) Treating cells with BFA alone also increased the apparent translation rate of a polypeptide that exactly comigrated with the 80-kDa protein. (d) In poliovirus-infected, BFA-treated cells, there was also a polypeptide whose migration mimicked that of the 80-kDa protein. Molecular mass markers are shown to the right of panel A.

mechanism (27) in poliovirus-infected cells even after the translation of most cellular proteins has been inhibited.

To determine whether the BFA-induced 80-kDa protein was a glucose-regulated protein such as grp78/BiP, two-dimensional gel electrophoresis was performed on [³⁵S]methionine-labeled extracts of cells deprived of glucose (Fig. 4). In the absence of glucose, the relative amount of an approximately 80-kDa protein was greatly increased (Fig. 4a and b). This 80-kDa protein comigrates with a polypeptide which is present in both poliovirus-infected and uninfected cells in the presence (Fig. 4d) of BFA. On the basis of its comigration with the 80-kDa protein whose synthesis is enhanced during glucose deprivation and the known ability of grp78/BiP mRNA to be translated during poliovirus infection (27, 36), the BFA-induced 80-kDa protein appears to be grp78/BiP.

BFA inhibits poliovirus RNA synthesis. The similarity of protein synthesis patterns between poliovirus-infected cells treated with guanidine and infected cells treated with BFA suggested that BFA, like guanidine, specifically inhibited viral RNA synthesis. To measure the effect of BFA on poliovirus RNA synthesis directly, infected cells were grown in the presence or absence of BFA and in the presence of 2 μg of actinomycin D per ml to inhibit cellular transcription. Synthesis of viral RNA was monitored by the accumulation of [^3H]uridine into trichloroacetic acid-insoluble material. As shown in Fig. 5a, poliovirus RNA accumulation in infected HeLa cells was maximal at 5 to 6 h postinfection. However, the incorporation of [^3H]uridine into RNA was reduced to levels identical with those of mock-infected cells (Fig. 5b) when 2 μg of BFA per ml was added at the initiation of poliovirus infection. Thus, poliovirus RNA synthesis early in the infectious cycle is inhibited by BFA.

Addition of BFA to poliovirus-infected cells 3 h postinfection also inhibited new poliovirus RNA synthesis (Fig. 5a), indicating that BFA could inhibit poliovirus RNA synthesis even after substantial amounts of poliovirus proteins were present. When unlabeled uridine in a 5,000-fold molar excess to [^3H]uridine was added at 3 h postinfection along with BFA, the amount of trichloroacetic acid-insoluble radioactivity did not diminish substantially. Therefore, BFA does not act by destabilizing previously synthesized poliovirus RNA. Furthermore, 2 μg of BFA per ml had little effect on host cell RNA synthesis over a 7-h period (Fig. 5b).

The inhibition of poliovirus infection by BFA is reversible. The inhibitory effects of BFA on ER-to-Golgi protein transport have been shown to be reversible; normal function is resumed within minutes of the removal of BFA from the culture medium (26). To examine the reversibility of BFA's effect on poliovirus replication, poliovirus-infected cells were incubated with BFA for 1, 2, or 3 h before the medium was replaced with drug-free medium. Figure 6a shows the effect of these treatments on virus yield throughout a single-cycle infection.

As was documented in Fig. 2a, the presence of BFA throughout the course of the poliovirus infectious cycle resulted in a greater than 10^5 -fold inhibition of poliovirus growth. When BFA was removed from the culture medium 1 h after infection, the growth curve of poliovirus did not differ significantly from that of the poliovirus infection in the complete absence of BFA. However when BFA was removed from the culture medium at 3 h postinfection, a 2-h delay in virus production was observed. An intermediate effect was observed when BFA was removed at 2 h postinfection. Despite the delays, by 8 h postinfection, the yields of poliovirus in samples treated with BFA early in infection were similar to that found in the untreated infection (Fig. 6a). Thus, the recovery of the poliovirus infections after BFA treatment was complete; the lag in virus production when BFA was present early in infection presumably reflects the necessity to establish physical structures within the cell necessary for viral RNA synthesis, the time needed to package newly synthesized RNAs into infectious virions, or both.

The data in Figure 6a show that although the inhibition of poliovirus growth was reversible, virus growth was very sensitive to BFA from 1 to 3 h postinfection. Figure 6b shows that when BFA was not added until 3 h postinfection, substantial amounts of virions were still produced. Therefore, by 3 h postinfection, the BFA-sensitive step(s) in viral RNA synthesis was at least partially completed. Since virion production continued in the presence of BFA, it seems

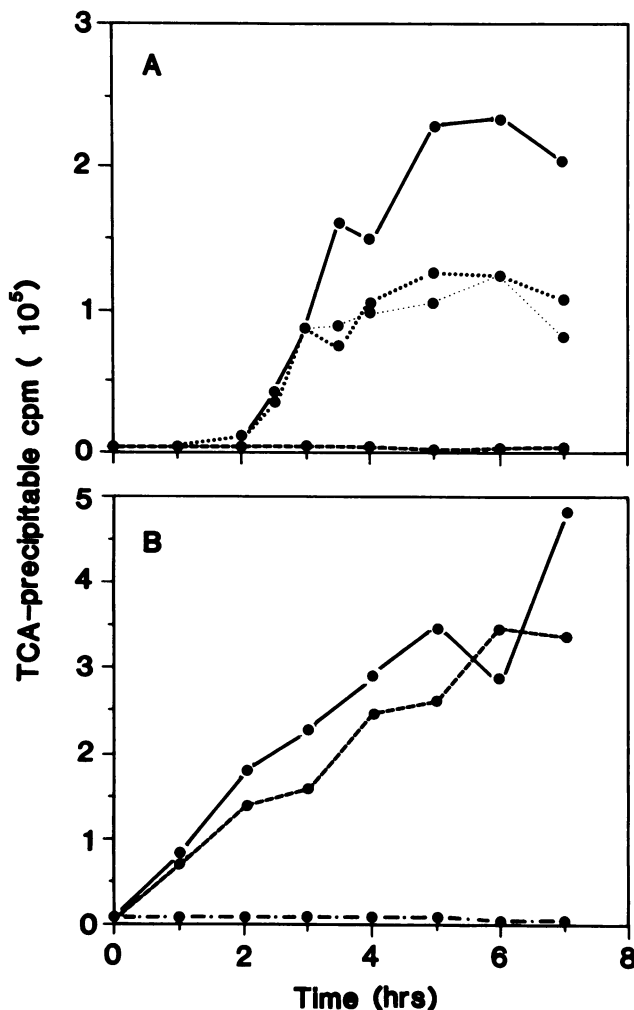


FIG. 5. Effect of BFA on RNA synthesis and stability in poliovirus-infected and uninfected HeLa cells. The incorporation of [^3H]uridine into RNA in infected and uninfected HeLa cells over time is shown. (A) RNA synthesis and accumulation in infected cells treated with no inhibitor (—), BFA added immediately after infection (---), BFA added 3 h after infection (····), and BFA and 0.1 M unlabeled uridine added 3 h after infection (-·-·). (B) RNA synthesis and accumulation in uninfected HeLa cells in the absence of BFA (—), in the presence of BFA (---), and in the presence of 5 μg of actinomycin D per ml (-·-·). TCA, trichloroacetic acid.

unlikely that later events in virus growth, like RNA packaging and virus assembly, were affected by BFA. When BFA was added 1.5 h postinfection, however, very little virus was produced. Taken together, the data in Fig. 6a and b argue that the most BFA-sensitive period in the poliovirus infectious cycle is from ~1 to 3 h postinfection.

BFA does not inhibit lipid synthesis in poliovirus-infected cells. Since the inhibition of lipid synthesis by cerulenin (18) has been reported to inhibit poliovirus replication 100-fold, one explanation of the inhibition of poliovirus RNA synthesis by BFA might be a similar inhibition of lipid synthesis. We monitored the effect of poliovirus infection and BFA treatment on the incorporation of [^3H]choline and [^{14}C]glycerol into HeLa cells; the rate of incorporation of [^3H]choline should indicate the rate of new synthesis of phospholipids,

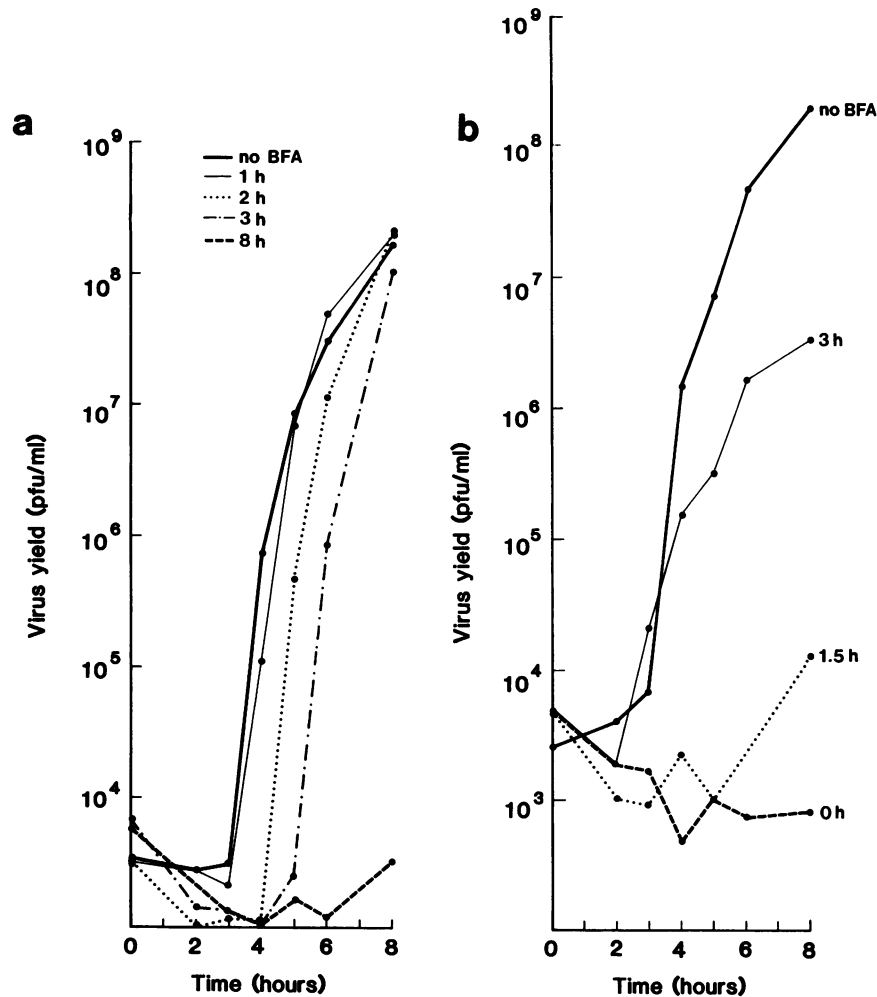


FIG. 6. Timing and reversibility of inhibition of poliovirus infection by BFA. Virus yields are shown during the course of single-cycle infections. (a) Growth curves of infected cells that were either not exposed to BFA or were exposed to BFA for the first 1, 2, or 3 h of infection or for the entire 8 h of the infectious cycle. (b) Growth curves of infected cells grown without BFA or with BFA added at the indicated times after infection.

and the rate of incorporation of [¹⁴C]glycerol should indicate new synthesis of all glycerol lipids. As can be seen in Fig. 7, the rate of incorporation of [³H]choline (Fig. 7a) into uninfected HeLa cells was not significantly affected by the presence of 2 μ g of BFA per ml although the rate of incorporation of [¹⁴C]glycerol (Fig. 7b) was inhibited slightly. This observation suggests that BFA produces a slight inhibition in the synthesis of a class of nonphospholipid glycerol lipids. However, it is clear from these data that the inhibition of poliovirus replication by BFA is not mediated by global inhibition of lipid synthesis.

In poliovirus-infected cells, there was a small, but significant, increase in the rate of incorporation of both [¹⁴C]glycerol and to a lesser extent of [³H]choline into lipid compared with uninfected cells (9; Fig. 7), suggesting that in addition to phospholipids, the synthesis of a particular, nonphospholipid class of glycerol lipids was preferentially stimulated in poliovirus-infected cells. Of greater importance here, however, was the observation that BFA inhibited the poliovirus-induced stimulation of lipid synthesis, i.e., the rate of

phospholipid synthesis in BFA-treated, poliovirus-infected cells returned to that of uninfected cells (Fig. 7a).

BFA does not inhibit adenovirus replication. BFA is sometimes described as an antiviral compound on the basis of its inhibition of replication of herpes simplex virus and Newcastle disease virus (42), both enveloped viruses, and its inhibition of the glycosylation of vesicular stomatitis virus G protein (16, 40). Recently, specific effects of BFA on the infectious cycles of several other enveloped viruses have been reported (12, 13, 45). The known effects of BFA on protein secretion are sufficient to explain the inhibition by BFA of any virus that requires newly synthesized glycosylated, secreted, or membrane-bound proteins during its infectious cycle. The inhibition of poliovirus infection by BFA cannot be explained in this simple manner, since poliovirus encodes no glycosylated or secreted proteins. However, we wanted to demonstrate that another virus whose metabolic requirements did not necessarily involve the protein secretion apparatus for infectivity was not inhibited by BFA.

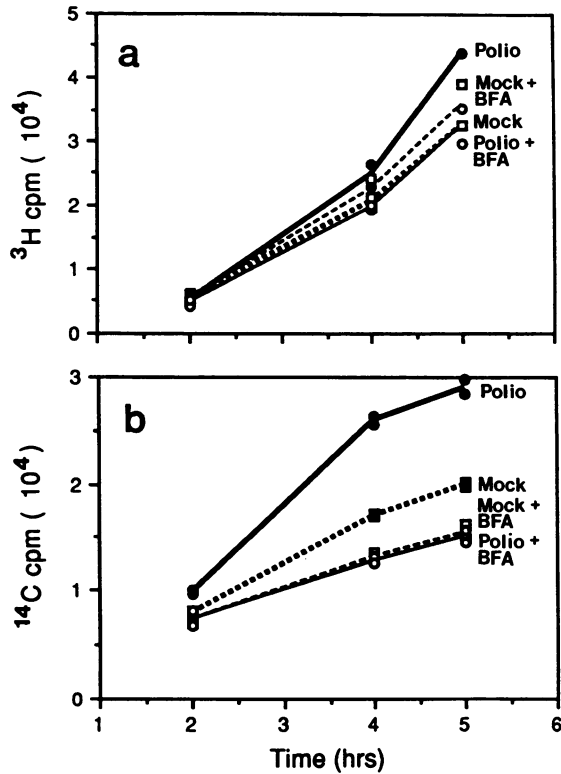


FIG. 7. Effect of BFA on lipid synthesis in infected and uninfected HeLa cells. Cells were labeled with 1 μ Ci of [³H]choline per ml (a) or 1 μ Ci of [¹⁴C]glycerol per ml (b) in the presence (---□---) or absence (---■---) of BFA. Lipid synthesis in poliovirus-infected cells is shown in the presence (—○—) and absence (—●—) of BFA.

Adenovirus is a nonenveloped, double-stranded DNA virus whose replication, transcription, and packaging occur in the nuclei of infected cells. Although several viral glycoproteins are produced during wild-type adenovirus infection, they are not required for viral growth in tissue culture (20). Infections of HeLa cells with adenovirus were performed in the presence and absence of BFA. For different 12-h intervals during a single-cycle adenovirus infection, 2 μ g of BFA per ml was added to the growth medium. The growth conditions for infections 1 to 5 and the yields of infectious adenovirus particles after 48 h as determined by plaque assay are shown in Fig. 8a. No significant difference in virus yield was observed when BFA was present during any of the 12-h intervals tested; the slight stimulation in virus yield in infection 5 was not considered significant.

It was possible that the adenovirus infections, like poliovirus (Fig. 6a), were inhibited by BFA early in infection but that recovery could occur when BFA was removed from the growth medium. To detect any possible lag in adenovirus growth in the presence of BFA at early times in the infectious cycle, DNA samples were prepared from infections 1 to 3 (Fig. 7a) at 0, 12, 24, 36, and 48 h postinfection. These DNA samples were immobilized to nitrocellulose filters and hybridized with ³²P-labeled adenovirus DNA probe to determine whether, at early times, adenovirus DNA synthesis was delayed in the presence of BFA. In Fig. 8b, an increase in adenovirus DNA can be seen between 0 and 12 h postinfection in the absence (infection 1) or presence (infection 2) of BFA during that same time period. Similarly, comparison of infections 1 and 3 reveals an increase in adenovirus DNA concentration from 12 to 24 h postinfection, whether BFA was present or absent during that time

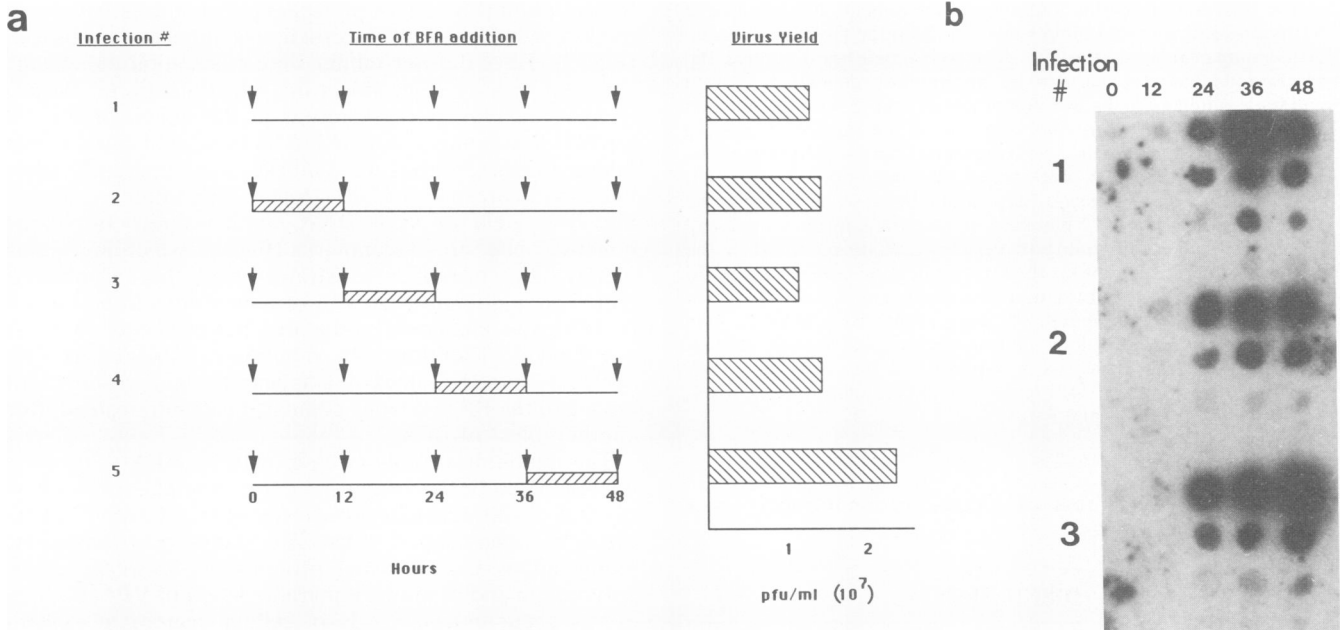


FIG. 8. Effect of BFA on the infectious cycle of adenovirus. (a) Diagram of experiment in which HeLa cells were infected with adenovirus in the absence (infection 1) or presence of 2 μ g of BFA per ml from 0 to 12 h postinfection (infection 2), 12 to 24 h postinfection (infection 3), 24 to 36 h postinfection (infection 4), or 36 to 48 h postinfection (infection 5). Time points at which aliquots of infected cells were sampled are shown by the arrows. Virus yield was determined after 48 h for each infection; the yield of virus is shown for each infection on the bar graph on the right. (b) Dot blots showing time course of adenovirus DNA synthesis for infections 1 to 3. The times (in hours) at which the samples were taken are shown at the top. Each sample is presented in successive 10-fold dilutions.

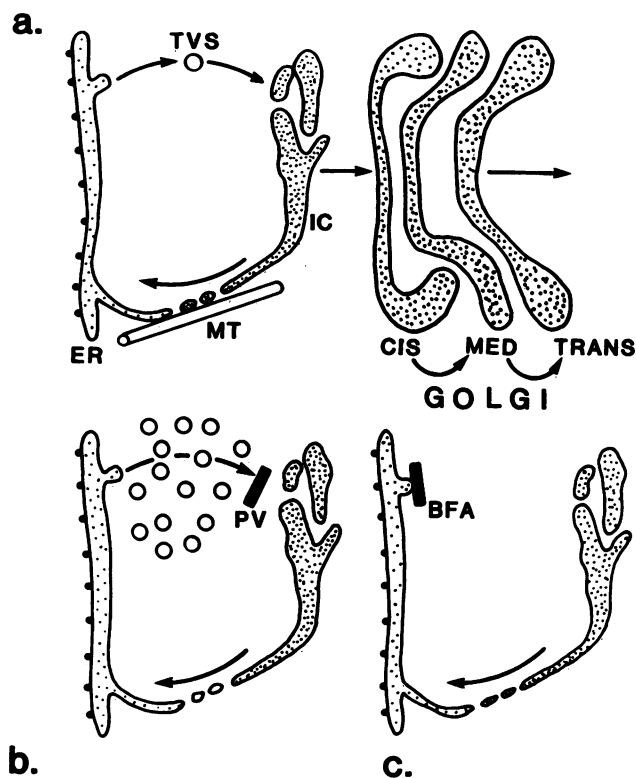


FIG. 9. A model for the inhibition of poliovirus RNA synthesis by BFA. (a) In a normal cell, transport of material from the ER to the Golgi apparatus involves the movement of transport vesicles from the ER to the intermediate compartment and then from the intermediate compartment to the cis compartment of the Golgi. Vesicular intermediates are also thought to mediate the transport of material from cis to medial and from medial to trans-Golgi compartments. In addition to this anterograde movement, there is also a retrograde pathway that moves material from the Golgi or intermediate compartment to the ER. This retrograde path, unlike the anterograde path, appears to be microtubule dependent. (b) In poliovirus-infected cells we hypothesize that a virally encoded activity is responsible for blocking one of the normal membrane fusion steps. For simplicity, we have shown the blocked step as the fusion of transport vesicles to the intermediate compartment (IC), although the blocked fusion step could be anywhere along the anterograde pathway. The blockage of membrane fusion would lead to the accumulation of transport vesicles that are recruited by the virus to form the scaffold for the viral replication complexes. (c) In BFA-treated cells, the formation of a number of transport vesicle types appears to be inhibited. We suggest that BFA short circuits the poliovirus-induced block of transport vesicle fusion, so that none of the types of vesicles required by the virus for RNA replication accumulate. Abbreviations: MT, microtubule; TVS, transport vesicles; PV, poliovirus.

period. We conclude that BFA does not inhibit adenovirus infection in HeLa cells.

DISCUSSION

Three to four hours after infection with poliovirus, human cells fill with small membranous vesicles (10, 11, 15). These vesicles are associated with the poliovirus replication complex (3-5). Their formation is thought to require the synthesis of poliovirus proteins (6, 19) and their appearance coincides with the commencement of poliovirus RNA synthesis

(4). Three lines of evidence argue that these vesicles are important in poliovirus RNA synthesis. First, newly synthesized poliovirus RNA molecules and several viral proteins known to be involved in viral RNA synthesis are physically associated with these vesicles (3-5). Second, preparations of membranes from infected cells, termed crude replication complexes, are capable of synthesizing poliovirus RNA *in vitro* (32, 41, 43). Third, cerulenin, an inhibitor of lipid synthesis, blocks poliovirus RNA synthesis and lowers the yield of virus 100-fold (18). While these data offer evidence that cellular membranes, and probably the small vesicles that appear in poliovirus-infected cells, are important for poliovirus RNA synthesis, they yield little insight into the derivation of these vesicles or the mechanism by which poliovirus infection induces their accumulation.

Small membranous vesicles are responsible for the movement of newly synthesized proteins from the ER to the Golgi apparatus and between compartments of the Golgi stack (34, 35). BFA was discovered over 30 years ago (38) and recently has been widely used to study membrane traffic and protein processing in eukaryotic cells (16, 25, 26, 29, 30, 40). After treatment of mammalian cells with BFA, ER to Golgi transport is rapidly inhibited. Because retrograde transport from the Golgi to the ER continues, the Golgi apparatus resorbs into the ER, resulting in a mixing of the two compartments in the presence of BFA (26, 29, 30). Retrograde transport is microtubule dependent, and the mixing of the ER and Golgi compartments in the presence of BFA can be inhibited by simultaneous treatment with nocodazole (25, 30).

The inhibition of poliovirus replication by BFA occurs at low concentrations and is not due to the inhibition of viral entry into the cell, the release of the poliovirus RNA (Fig. 2 and 3a), or the translation of viral RNA either early or late in the infectious cycle (Fig. 3). BFA's effect also does not appear to be related to nonspecific effects on host cell transcription (Fig. 5b) or phospholipid synthesis, although a modest reduction of all glycerol lipid synthesis was observed (Fig. 7). BFA did not inhibit viral translation either early (Fig. 3a) or later (Fig. 3b) in the infectious cycle. Surprisingly, a cellular protein present in the lumen of the ER, *grp78/BiP*, not only continued to be translated in poliovirus-infected cells (36), but its synthesis was apparently stimulated in the presence of BFA (Fig. 4). BFA had no effect on the viral yield or viral DNA synthesis rate of another nonenveloped virus, adenovirus (Fig. 8), which has a completely different replication strategy from that of poliovirus (20). The inhibition of growth of poliovirions in HeLa cells by BFA was unaffected by the presence of nocodazole (data not shown). Therefore, the inhibition of poliovirus RNA synthesis by BFA does not appear to be a result of the mixing of the ER and Golgi compartments but, instead, from another effect of BFA.

The inhibition of poliovirus growth by BFA suggests an unanticipated link between the cytopathic effect of poliovirus and the intracellular protein transport pathway. Poliovirus is a nonenveloped virus. The known posttranslational modifications of poliovirus proteins are cleavage of the polypeptide and N-terminal myristoylation of VP4 (14); both events occur independently of cellular membrane systems (14, 33). Therefore, poliovirus infection should not display a strict requirement for the protein glycosylation and secretion functions of the ER and Golgi apparatus. We suggest that BFA may inhibit poliovirus growth by inhibiting the formation of a specific class of vesicles required in infected cells for poliovirus RNA synthesis (Fig. 9). As discussed above,

the effects of BFA on protein traffic are most consistent with inhibition of the formation of vesicles involved in ER-to-Golgi and intra-Golgi traffic (25, 26, 29, 30).

The greater stimulation of total glycerol lipids than of phospholipid (Fig. 7) during poliovirus infection and the inhibition of this stimulation by BFA, together with the inhibitory effects of the lipid synthesis inhibitor cerulenin (18) on poliovirus replication, suggest that a particular class of glycerol lipid may be utilized in the synthesis of the poliovirus-induced vesicles. The inhibition of poliovirus infection by BFA even in the presence of nocodazole argues that fragmentation of the Golgi apparatus and its resorption into the ER in the presence of BFA are not responsible for the inhibition of viral RNA synthesis.

In addition to the known specificity of BFA, several findings presented here are consistent with the idea that the BFA-sensitive step in poliovirus RNA synthesis is the formation of the virus-induced vesicles. First, the time (1 to 3 h postinfection) at which the inhibition of poliovirus growth by BFA is most pronounced (Fig. 6) is the time at which the poliovirus-induced vesicles first form (4). Second, after the removal of BFA from poliovirus-infected cells, it takes approximately 2 h before the production of virions begins (Fig. 6). This surprisingly long lag time may include the time needed to synthesize vesicular structures necessary for viral RNA synthesis.

The major prediction of the model shown in Fig. 9 is that poliovirus encodes an activity or takes advantage of an activity inherent to cells that blocks membrane fusion and thereby leads to the accumulation of vesicles derived from the cell's membranous compartments. Our model predicts that BFA inhibits poliovirus RNA synthesis by blocking the formation of these vesicles (Fig. 9c). This model also suggests that poliovirus, not needing the glycosylation or secretion functions of the host cell, usurps the host's membrane system for its own highly specialized needs. Of course, it is also possible that BFA directly inhibits a particular host protein or proteins required for poliovirus replication. Work is in progress to distinguish between these models for BFA's effect on poliovirus replication.

ACKNOWLEDGMENTS

The contributions of L. A. Maynell and K. Kirkegaard to this work are equivalent.

We are grateful to Tami Kano-Sueoka and Harold Fisk for advice on lipid synthesis measurements and to Paul Melançon, Peter Sarnow, and Graham Warren for advice, encouragement, and reagents during the course of these experiments. L.A.M. wishes to thank Louisa M. Morrissey and Benjamin Young for moral support and use of computer facilities. We thank Joseph Dent, Paul Melançon, and Peter Sarnow for critical reading of the manuscript.

M.W.K. was supported by the Pew Biomedical Scholars Program and grant DCB 89-0522 from the NSF, and K.K. was supported by NIH grant AI-25166 and by the David and Lucile Packard Foundation. K.K. is an assistant investigator of the Howard Hughes Medical Institute.

ADDENDUM IN PROOF

Recent work from several groups has demonstrated inhibitory effects of BFA on additional intracellular membrane traffic pathways, specifically on functions of early endosomes and the trans-Golgi network (S. A. Wood, J. E. Park, and W. J. Brown, *Cell* 67:591-600, 1991; J. Lippincott-Schwartz, L. Yuan, C. Tipper, M. Amherdt, L. Orci, and R. D. Klausner, *Cell* 67:601-616, 1991), affecting processes such as transcytosis and lysosomal targeting but not endocytosis

(W. Hunziker, J. A. Whitney, and I. Mellman, *Cell* 67:617-627, 1991; Lippincott-Schwartz et al., *Cell*, 1991). Thus, in addition to the ER and the Golgi apparatus, these organelles or structures derived from them are also candidates for involvement in the BFA-sensitive step of poliovirus RNA synthesis.

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