Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2291–2295, April 1982
Cell Biology

Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of α_2-macroglobulin

(viral infection/antiviral agents/inhibitors of endocytosis)

RICHARD SCHLEGEL, ROBERT B. DICKSON, MARK C. WILLINGHAM, AND IRA H. PASTAN

Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Communicated by J. E. Rall, December 23, 1981

ABSTRACT **The entry of many animal viruses into their host cells often proceeds via a specialized internalization pathway involving clathrin-coated regions of the plasma membrane. We have examined the effect of dansylcadaverine and amantadine on the entry of vesicular stomatitis virus (VSV) into mouse cells. Both compounds inhibit VSV entry as determined by fluorescence and electron microscopy, ^{3}H-labeled VSV uptake, and VSV-dependent RNA synthesis assays. They also inhibit the uptake of α_2-macroglobulin, a protein that binds to specific membrane receptors and follows the same route of internalization. Dansylcadaverine is 20-fold more potent than amantadine in blocking virus and α_2-macroglobulin uptake. One cellular target for both of these amine-containing compounds appears to be the clustering of membrane-bound ligands or particles in clathrin-coated pits.**

Investigations of viral penetration into host cells are complicated by the observation that viruses may have more than one entry mechanism. Studies with vesicular stomatitis virus (VSV) have focused on two possible pathways of infection: viropexis (entry within endocytic vesicles) and direct fusion with the plasma membrane. Although direct fusion of VSV with the plasma membrane has been observed (1, 2), most evidence favors the hypothesis that VSV enters cells via an endocytotic mechanism (3, 4, 5) that is mediated by coated pits. Recent investigations with Semliki Forest virus have shown that this virus also undergoes similar endocytosis (6, 7).

Coated pits are the site of entry for many hormones and protein ligands that enter cells via receptor-mediated endocytosis. This process involves the binding of ligand to specific membrane receptors, the association of the ligand–receptor complexes with clathrin-coated regions of the plasma membrane, and finally the endocytosis of these complexes into cytoplasmic vesicles termed "receptosomes" (8). Recent electron microscopic studies have shown that VSV and α_2-macroglobulin (α_2M) can enter cells together in the same coated pit and receptosome, implying a similar endocytosis mechanism (9).

Dansylcadaverine is an effective inhibitor of receptor-mediated endocytosis; it reduces the cellular internalization of α_2M (10), triiodothyronine (11), insulin (unpublished data), low density lipoprotein (unpublished data), and epidermal growth factor (12). The current study demonstrates that dansylcadaverine also prevents the internalization of VSV into susceptible mouse cells, apparently by blocking the entry of virions into coated-pit structures. Amantadine, a documented antiviral compound, can also inhibit receptor-mediated endocytosis of α_2M at concentrations comparable to those required for its antiviral activity against VSV. Our results demonstrate that agents which inhibit the sequestration of VSV in coated pits also inhibit viral infection. In addition, the data provide support for endocytosis being the primary functional route of VSV infection.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Cultures. Swiss 3T3 mouse cells were grown in Dulbecco–Vogt modified Eagle's medium supplemented with 10% calf serum. For experiments, 10^5 cells were seeded into 35-mm plastic dishes and incubated at 37°C for 3 days (until confluent).

Virus. Purified VSV (Indiana strain) was obtained from J. Brown at the University of Virginia. The virus was grown in BHK (baby hamster kidney) cells and purified by sucrose velocity and density gradients to approximately 1 mg of VSV protein per ml. ^{3}H-Labeled VSV was prepared by infecting BHK cells in the presence of [^{3}H]uridine at 5 μCi/ml (1 Ci = 3.7×10^{10} becquerels). Subsequent to cell lysis, the virus was purified as above. The specific activity of the VSV preparation was 3,400 cpm/μg of VSV protein or approximately 3,400 cpm per 2×10^9 VSV particles.

Virus Infection of Swiss 3T3 Mouse Cells. Viral infections were performed by aspirating the culture medium, replacing it with 1.0 ml of fresh medium, and incubating the cultures at 4°C for 15 min. Then 5–20 μl of purified virus stock was added to each culture dish and the dishes were gently rocked at 4°C for 60 min. The multiplicity of infection was approximately 1,000 VSV plaque-forming units (PFU) per cell. The efficiency of VSV binding at 4°C for 60 min was 1% of the viral input (determined with [^{3}H]uridine-labeled VSV). The actual multiplicity of infection was 10 PFU per cell under the conditions stated and the approximate PFU-to-particle ratio was 1:100. Inhibitors were added during the final 5 min of the 4°C incubation period. Cultures were transferred to 37°C for 10 min, after which they were washed twice with 2.0 ml of fresh medium. The cultures were then maintained in Dulbecco's medium with 10% calf serum containing 2% heated rabbit antiserum to the VSV G protein. One percent rabbit anti-G protein antiserum had been found sufficient to totally neutralize the cell-bound VSV present on Swiss 3T3 cells after the 60-min adsorption period.

Assay of Viral RNA Synthesis. VSV-dependent RNA synthesis was used to estimate VSV entry into the cell cytoplasm. This procedure has been described previously (13). Immediately after infection with VSV, actinomycin D (5 μg/ml) was added for 30 min. Cultures were then labeled with [^{3}H]uridine (New England Nuclear) at 10 μCi/ml for 1–2 hr at 37°C. After the supernatant had been decanted, the cultures were washed twice with phosphate-buffered saline (P_i/NaCl) and then fixed with cold (4°C) 10% trichloroacetic acid for 30 min. The culture plates were washed twice with cold 10% trichloroacetic acid and

Abbreviations: α_2M, α_2-macroglobulin; VSV, vesicular stomatitis virus; PFU, plaque-forming units; P_i/NaCl, phosphate-buffered saline.

three times with distilled water. The trichloroacetic acid precipitate was then solubilized with 2.0 ml of 1 M NaOH and neutralized with 2.0 ml of 1 M HCl; the radioactivities of 0.5-ml aliquots were measured in a liquid scintillation counter.

Immunofluorescence. Uptake of VSV into mouse cells was detected by indirect immunofluorescence using rabbit antiserum prepared against VSV G protein. Swiss 3T3 cells were infected for 60 min at 4°C with 5,000 VSV PFU per cell. Inhibitors were added and the temperature was raised to 37°C for 10 min to allow viral uptake. Externally bound VSV virions were removed with 0.02% EDTA and 0.125% trypsin. Experiments with ^{3}H-labeled VSV indicate that this trypsinization procedure removes 95% of surface-bound VSV (results not shown). The cells were then centrifuged, replated in medium, and incubated at 37°C for 25–30 min until they had reattached to the plastic surface. The cultures were washed gently with P_i/NaCl and fixed with 80% (vol/vol) acetone for 10 min at 4°C. The acetone was aspirated and the plates were air dried. Plates were rinsed twice with P_i/NaCl and incubated with affinity-purified rabbit anti-G protein antibody at 100 μg/ml in a mixture containing normal goat globulin at 500 μg/ml. After 30 min at room temperature, the plates were washed five times with P_i/NaCl and incubated with a 1:25 dilution of rhodamine-labeled goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) for another 30 min at room temperature. Finally, the plates were washed three times with P_i/NaCl and two times with distilled water, mounted in a 9:1 (vol/vol) glycerol/P_i/NaCl solution, and examined by epifluorescence microscopy. Photographs were taken on Polaroid 107 film with 5-sec exposures.

^{3}H-Labeled VSV Uptake. Swiss 3T3 mouse cells were grown to confluence in 100-mm plastic dishes. The culture medium was discarded and 1.0 ml of fresh medium (with and without inhibitors) was added. Thirty microliters of ^{3}H-labeled VSV stock (1 μg of VSV protein per μl; 3,400 cpm/μg of VSV protein) was added to each 100-mm plate. The multiplicity of infection was approximately 50 PFU per cell. The cultures were maintained at 37°C for 30 or 60 min, after which they were rinsed twice with a 4°C trypsin/EDTA solution (P_i/NaCl containing 0.125% trypsin and 0.02% EDTA). Finally, the cells were incubated in the trypsin/EDTA solution for 20 min at 37°C, centrifuged at 2,000 × *g* for 5 min, and solubilized in 1 M NaOH. Radioactivities of aliquots were measured by a liquid scintillation technique. All assays were performed in duplicate.

Chemicals. Dansylcadaverine (Sigma) was prepared fresh for each experiment. The stock solution (5 mM) was prepared in P_i/NaCl that was slightly acidified with 0.1 M HCl. Amantadine and rimantadine (a gift of Du Pont) were dissolved in P_i/NaCl and kept as 0.5 M stock solutions at 4°C.

Electron Microscopy: Clustering of α_2M in Coated Pits. The distribution of α_2M on cell surfaces was studied after incubations of cells with α_2M at 0°C. Cells with or without inhibitors were incubated with α_2M at 250 μg/ml at 0°C for 45 min. The cells were washed twice with P_i/NaCl with or without inhibitors at 0°C and fixed with glutaraldehyde in P_i/NaCl at 0°C. The cells were then incubated with affinity-purified rabbit anti-α_2M antibody (8) (50 μg/ml) for 15 min at 23°C, washed, and incubated with (1:20 dilution) peroxidase-labeled goat anti-rabbit antibody (Cappel Laboratories) for 15 min at 23°C. After the cells had been rewashed, they were again fixed in 1% glutaraldehyde, incubated with diaminobenzidine/H_2O_2 substrate solution (8), postfixed in OsO_4, dehydrated with ethanol, and embedded *in situ* in Epon 812. Thin sections were counterstained with lead citrate and viewed at 50 kV with a Hitachi HU-12A electron microscope.

Electron Microscopy: Sequestration of VSV in Coated Pits. Swiss 3T3 cells were incubated for 5 min at 37°C in medium with dansylcadaverine or amantadine. VSV was then added at 5,000 PFU per cell and the cultures were maintained at 37°C for 10 min. The cultures were then washed in P_i/NaCl and immediately fixed in 2% (wt/vol) glutaraldehyde and processed for electron microscopy. Control cultures were treated in the same fashion in the absence of any inhibitors. Thin sections were counterstained with uranyl acetate and lead citrate. VSV uptake was quantitated by randomly examining the location of all VSV particles associated with cells and identifying their location on the free cell surface, in coated pits, or in receptosomes. The total number of viral particles counted was 413 in the control, 274 for dansylcadaverine, and 320 for amantadine.

Electron Microscopy: Clustering and Internalization of VSV and α_2M·Gold. Swiss 3T3 cells were incubated at 4°C with α_2M·gold (9) (30 μg/ml of protein) and VSV (5,000 PFU per cell) simultaneously for 2 hr. After washing in serum-free medium at 4°C, the cells were warmed to 37°C for 2 min and then fixed in 2% glutaraldehyde and processed for electron microscopy (8). At this time, ligands can be found both clustered in coated pits and internalized into receptosomes. Incubation with a 20-fold excess of unlabeled α_2M prevented any labeling with α_2M·gold (9).

RESULTS

Fluorescence Visualization of Virus Internalization. Virus uptake into Swiss 3T3 mouse cells was evaluated by an indirect immunofluorescence assay. Swiss 3T3 cells were exposed to VSV at a multiplicity of 5,000 PFU per cell. Virus adsorption was performed at 4°C for 60 min, after which dansylcadaverine was added to a final concentration of 200 μM. The cultures were kept at 4°C for an additional 5 min and then changed to 37°C for 10 min to permit viral uptake. The cultures were trypsinized to remove externally bound VSV virions and replated in fresh medium at 37°C. After the cells had reattached to the substratum (30 min later), they were washed with P_i/NaCl, fixed with acetone, air dried, and allowed to react with affinity-purified rabbit anti-G protein antibody and then with rhodamine-labeled goat anti-rabbit IgG. The cells were photographed with epifluorescence microscopy. The results are shown in Fig. 1. Mock-infected cells (Fig. 1*A*) did not stain for the virus-specific G protein, whereas cells infected with VSV (Fig. 1*B*) contained significant amounts of intracellular G protein, indicating the internalization of VSV particles. VSV-exposed cells that had

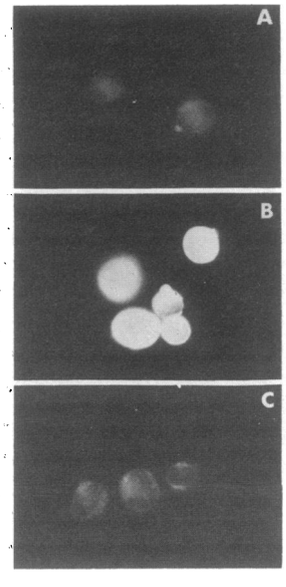

FIG. 1. Immunofluorescent detection of VSV uptake by Swiss 3T3 cells. Swiss 3T3 cells were bound with VSV at 4°C for 60 min (multiplicity of infection = 5,000). The cells were then transferred to 37°C for 10 min in the absence or presence of 200 μM dansylcadaverine. After removal of noninternalized VSV with trypsin/EDTA, intracellular viral G protein was stained. (*A*) Uninfected cells, (*B*) VSV-infected cells, (*C*) VSV-infected cells plus 200 μM dansylcadaverine. (×500.)

been treated with dansylcadaverine contained little or no G protein (Fig. 1*C*), suggesting that dansylcadaverine had prevented VSV uptake during the 37°C incubation period. Similar results were obtained with 5 mM amantadine (data not shown). Although the immunofluorescence results indicated that dansylcadaverine and amantadine could prevent virus uptake, it was not possible to quantify the inhibitory effect with this assay or eliminate the possibility that dansylcadaverine affected viral processes subsequent to internalization. Therefore, two additional techniques were used.

Quantitation of VSV-Dependent RNA Synthesis. VSV was bound to Swiss 3T3 cells by incubation at 4°C for 60 min. Different concentrations of dansylcadaverine were then added to the cell cultures, which were incubated for an additional 5 min at 4°C. The cultures were then transferred to a 37°C incubator for 10 min, after which the medium was removed and fresh Dulbecco's medium (minus the inhibitors) was added containing anti-G protein antibody to neutralize noninternalized virus (see *Materials and Methods*). Virus internalization was quantified by measuring VSV-specific RNA synthesis in the infected cells at 1.0–2.0 hr after infection. Both dansylcadaverine and amantadine reduced VSV-dependent RNA synthesis in Swiss 3T3 cells (Fig. 2). Dansylcadaverine was 20-fold more potent than amantadine. The half-maximal inhibitory concentrations of dansylcadaverine and amantadine were 50 μM and 1 mM, respectively. The effect of dansylcadaverine was reversible. When neutralizing antiserum was eliminated from the infection protocol, infected and dansylcadaverine-treated cultures had identical values for viral RNA synthesis (data not shown).

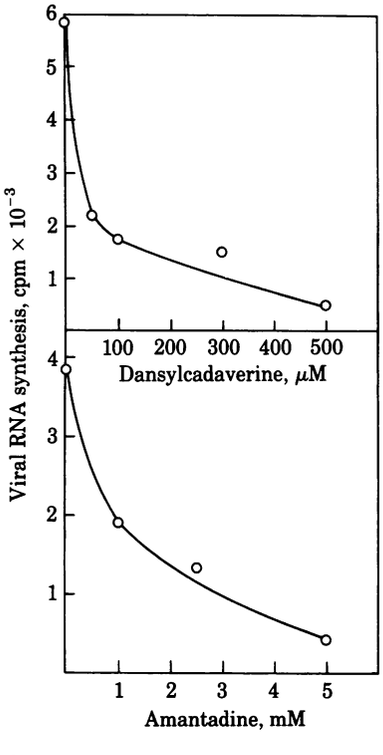

FIG. 2. Reduction of VSV-dependent RNA synthesis by dansylcadaverine and amantadine. Confluent monolayers of Swiss 3T3 cells were infected as described in the text with VSV in the presence and absence of dansylcadaverine or amantadine. Noninternalized virus was neutralized with anti-G protein antibody. One to 2 hr after infection, [^{3}H]uridine incorporation into macromolecules (in the presence of actinomycin D) was used to measure VSV-dependent RNA synthesis. When VSV-neutralizing antibody was omitted, control levels of VSV-dependent RNA synthesis were observed in the inhibitor-treated cultures, indicating the reversibility of dansylcadaverine and amantadine. In the absence of actinomycin D and VSV, cellular RNA synthesis was equivalent in control and drug-treated cultures. There was no evidence of cytotoxicity with this experimental protocol.

Table 1. Inhibition of ^{3}H-labeled VSV uptake by dansylcadaverine and amantadine

Additions to culture	^{3}H-Labeled VSV uptake per plate	
	cpm	%
VSV	2666, 2658	100
VSV + amantadine	406, 348	14
VSV + dansylcadaverine	268, 222	9

Confluent monolayer cultures of Swiss 3T3 cells were infected with 30 μl of ^{3}H-labeled VSV stock. Simultaneously, dansylcadaverine and amantadine were added to final concentrations of 200 μM and 5 mM, respectively. The cultures were incubated at 37°C for 60 min, washed, and trypsinized to remove noninternalized virus. Internalized virus was estimated by solubilizing the cells in 1 M NaOH and measuring the radioactivity in aliquots by liquid scintillation. The amount of ^{3}H-labeled VSV bound to Swiss 3T3 cultures kept at 4°C (468 cpm per plate) was subtracted from the measured values to calculate all the data in the table. Results are given for duplicate experiments. Percents are VSV internalized compared to the 37°C control cultures.

^{3}H-Labeled VSV Uptake. Viral uptake was also measured with internally labeled ([^{3}H]uridine) VSV. Confluent cultures of Swiss 3T3 cells were infected with 30 μl of concentrated ^{3}H-labeled VSV stock (1 μg of VSV protein per μl; 3,400 cpm/μl) for 60 min at 4°C or 37°C in the presence and absence of dansylcadaverine or amantadine. Cultures were trypsinized to remove noninternalized virus and solubilized in NaOH, and their contents of radioactivity were determined in a liquid scintillation counter. The results are shown in Table 1. Both dansylcadaverine and amantadine reduced the uptake of VSV to approximately 10% of control. This degree of inhibition is directly comparable to the results obtained with the VSV-dependent RNA synthesis assay.

Previous studies have shown that amantadine has no effect on viral binding to host cells (14, 15). The same was true for dansylcadaverine. Monolayers were infected with 30 μl of ^{3}H-labeled VSV stock at 4°C for 30 min, in the absence and presence of dansylcadaverine at 250 μg/ml. After one wash with P_i/NaCl, the control and dansylcadaverine-tested cultures bound 1,350 cpm and 1,260 cpm, respectively. After five washes with P_i/NaCl, the binding was reduced to 280 cpm and 250 cpm. Dansylcadaverine did not affect the amount of ^{3}H-labeled VSV that was either loosely or tightly bound to the cells.

Inhibition of α_2M Uptake. Dansylcadaverine, amantadine, and rimantadine (a structural analogue of amantadine) were evaluated for their ability to inhibit the internalization of the protein ligand α_2M. Confluent Swiss 3T3 cells were washed with serum-free medium to remove exogenous α_2M and then

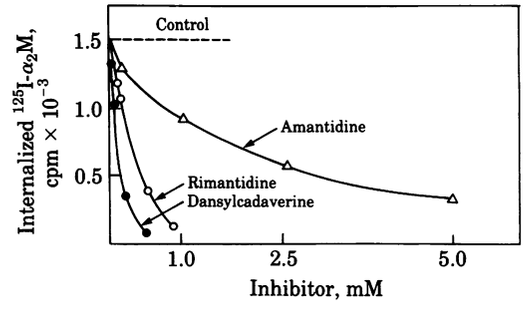

FIG. 3. Inhibition of ^{125}I-α_2M uptake by amantadine, rimantadine, and dansylcadaverine. Confluent monolayers of Swiss 3T3 cells were exposed to ^{125}I-α_2M at 50 μg/ml for 45 min at 37°C, with and without inhibitor. Uptake of α_2M was measured as described (16).

incubated with ^{125}I-labeled α_2M (^{125}I-α_2M) at 0.5 μg/ml for 45 min at 37°C, with and without inhibitor. Internalization of α_2M was quantified by utilizing an acid protease digestion technique (16). Fig. 3 presents dose–response curves for the inhibition of uptake of radiolabeled ligand by dansylcadaverine, amantadine, and rimantadine; the half-maximal inhibitory concentrations were 150 μM, 2.5 mM, and 0.5 mM, respectively. As with VSV uptake, dansylcadaverine was approximately 20-fold more potent an inhibitor than amantadine.

Inhibition of Ligand Sequestration in Clathrin-Coated Pits. Normally, both VSV and α_2M are internalized via clathrin-coated pit regions of the plasma membrane (9). Thus, when Swiss 3T3 cells were exposed simultaneously to VSV (5,000 PFU) and α_2M bound to colloidal gold (30 μg/ml), both ligands were observed within the same coated pit structures (Fig. 4*A*) and were internalized into the same receptosome (Fig. 4*B*). However, the addition of dansylcadaverine, amantadine, and rimantadine drastically perturbed this uptake mechanism. Fig. 4 compares representative sections of VSV-infected cells (Fig. 4*C*), VSV-infected cells with 100 (Fig. 4*D*) or 500 μM (Fig. 4*E*) dansylcadaverine, and VSV-infected cells with 1.0 mM rimantadine (Fig. 4*F*). VSV virions were found in coated pit structures of the plasma membrane in the absence of the inhibitors. In the presence of the inhibitors, most of the virus remained associated with noncoated plasma membrane. A quantitative electron microscopy study of viral uptake in the presence of 250 μM dansylcadaverine or 2.5 mM amantadine was performed. Random cells were evaluated for the number of VSV particles observed on the plasma membrane, in coated pits, or in intracellular endocytic vesicles (Fig. 5). Dansylcadaverine and amantadine decreased intracellular VSV by 64% and 41%, respectively. These data are comparable to the results of the viral RNA synthesis assay, which showed a 74% inhibition for dansylcadaverine and a 60% inhibition for amantadine at the same drug concentrations. The 20% difference observed between the EM and RNA synthesis experiments with amantadine might derive either from EM sampling error or from additional lysosomal effects of amantadine that could contribute to the inhibition of VSV appearance in the cytoplasm. However, the predominant mode of inhibition for dansylcadaverine and amantadine in Swiss 3T3 cells appears to be a reduction in viral endocytosis. The number of VSV particles in coated pits was also reduced by dansylcadaverine or amantadine treatment, although the quantities observed were very small. Recent studies have indicated that an antiviral activity of dansylcadaverine might be due to its effect upon lysosome function (17). The half-maximal inhibitory concentration for these apparent lysosomotropic effects was 400 μM. In our experiments, 50 μM dansylcadaverine inhibited VSV uptake by 50% under conditions in which lysosomal pH appears to be unaffected (18).

A similar blockade was noted for the uptake of α_2M. Swiss 3T3 cells were incubated with α_2M (250 μg/ml) for 45 min at 4°C. Various concentrations of amantadine and rimantadine were present during the incubation period. The cells were then washed and fixed, and the α_2M was located by using horseradish peroxidase labeling. Fig. 6 illustrates the profound inhibition of α_2M appearance in coated pits by either amantadine (Fig. 6*C*) or rimantadine (Fig. 6*D*). In the absence of inhibitors, α_2M was easily detected in coated pits by peroxidase labeling (Fig. 6*B*).

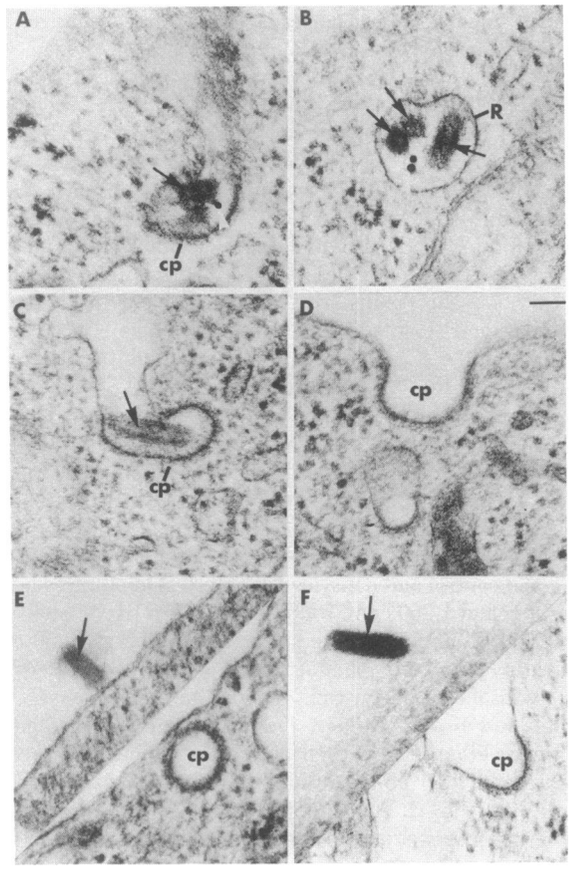

FIG. 4. Co-internalization of VSV and α_2M·gold and the inhibition of VSV internalization by dansylcadaverine and rimantadine. Swiss 3T3 cells were simultaneously exposed to VSV (multiplicity of infection = 5,000) and α_2M·gold (30 μg/ml). After a 2-min incubation at 37°C, the cells were fixed and processed for electron microscopy. An analogous infection with VSV alone was performed in the presence and absence of 100 and 500 μM dansylcadaverine and 1 mM rimantadine. (*A*) VSV (black arrows) and α_2M·gold (white arrowheads) in the same coated pit (cp); (*B*) VSV (black arrows) and α_2M·gold (white arrowheads) within same receptosome; (*C*) infected Swiss 3T3 cell with VSV in coated pit; (*D*) vacant coated pit of infected Swiss 3T3 in the presence of 100 μM dansylcadaverine; (*E*) vacant coated pit of infected Swiss 3T3 cell in the presence of 500 μM dansylcadaverine. Membrane-bound VSV is indicated by the black arrow. (*F*) Vacant coated pit of infected Swiss 3T3 cell in the presence of 1 mM rimantadine. Black arrow indicates membrane-bound VSV on the same cell. (Bar = 0.1 μm.)

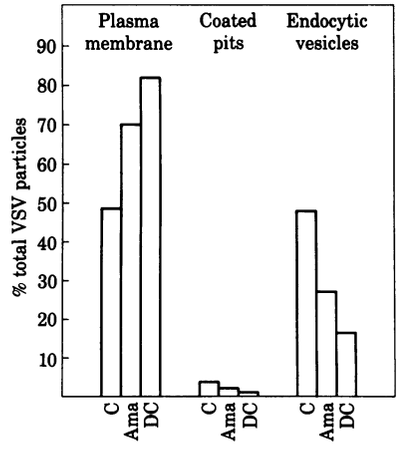

FIG. 5. Quantitation of VSV endocytosis by electron microscopy. Swiss 3T3 cells were infected with approximately 5,000 PFU per cell for 10 min at 37°C. Infections were performed in the absence of inhibitors (C, control) or in the presence of 250 μM dansylcadaverine (DC) or 2.5 mM amantadine (Ama). The cells were washed, fixed, and prepared for electron microscopy. Random cells were evaluated for the presence of VSV particles and their location was described as plasma membrane, coated pit, or endocytic vesicle.

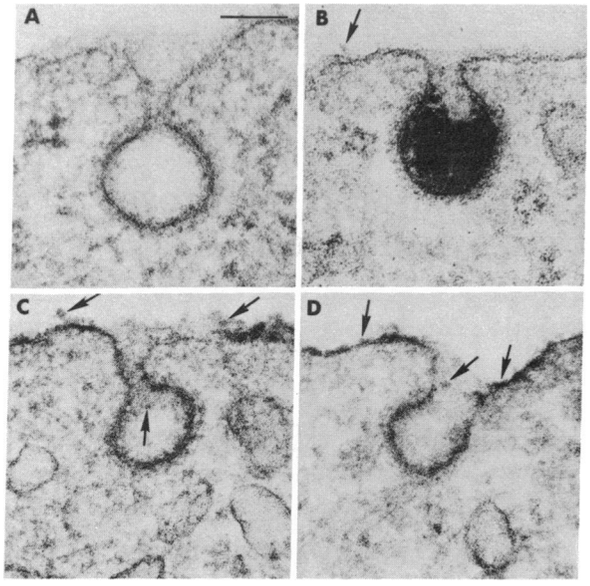

FIG. 6. Appearance of α_2M in coated pits and its inhibition by dansylcadaverine and amantadine. Swiss 3T3 cells were incubated with α_2M at 250 μg/ml at 4°C, with and without inhibitor. Electron microscopic localization of α_2M was performed by using a peroxidase staining technique. (*A*) Empty coated pit of control (no α_2M) cell; (*B*) α_2M localized to coated pit, no inhibitor present; (*C*) α_2M bound diffusely to the plasma membrane in the presence of 5 mM amantadine; (*D*) α_2M bound to plasma membrane in the presence of 1 mM rimantadine. Arrows illustrate bound α_2M. (Bar = 0.1 μm.)

DISCUSSION

Dansylcadaverine, amantadine, and rimantadine effectively block the uptake of VSV and α_2M into mouse cells. The inhibition of internalization is a reversible phenomenon, which can be achieved with relatively low concentrations of dansylcadaverine (50 μM). The blockade of virus entry by these inhibitors appears to occur at the stage of viral association with the clathrin-coated pit regions of the plasma membrane, and it agrees well with the findings that dansylcadaverine inhibits α_2M uptake at this same step. That dansylcadaverine and amantadine inhibit uptake of both virus and α_2M may indicate that the internalization of these ligands is functionally, as well as morphologically, similar. In support of this hypothesis, we have recently delineated a saturable binding site of VSV on the surface of Vero cells (unpublished results). The precise mechanism of the dansylcadaverine inhibition is unknown. However, it does appear to be selective for receptor-mediated processes because the adsorptive endocytosis of concanavalin A and fluid-phase pinocytosis of inulin are unaffected (unpublished results).

The specific step of viral infection that is blocked by amantadine has been somewhat controversial. Whereas some investigations have demonstrated an inhibition of viral penetration into the host cell (14), others have shown the cellular uptake of virus to be unperturbed (15, 19). These differences could arise either from the different viruses, host cells, and methods used or from the concentrations of amantadine employed. In general, influenza A virus is among the most sensitive of animal viruses to amantadine (19), although rubella virus (20), Sendai virus (21), lymphocytic choriomeningitis virus (14), and fowl plague virus (15) can also be inhibited. VSV is not highly sensitive to amantadine and, in our experiments, 1 mM amantadine was required to produce significant effects on virus-mediated cell killing. At these concentrations, we have shown that viral internalization is inhibited. Interestingly, it requires approximately 0.5 and 1.0 mM amantadine to produce strong inhibition of influenza virus protein and RNA synthesis (22), but marked inhibitory effects on viable virus production or hemagglutinin titers have been reported with concentrations as low as 125 μM. It is possible that some viruses might have multiple steps in their "life-cycle" that are inhibited by amantadine, thereby increasing their sensitivity to the drug. Lymphocytic choriomeningitis virus, for example, appears to be sensitive to amantadine before as well as after its penetration into host cells (14). Because amines can become concentrated in lysosomes (23) or other acidic organelles, they may also be able to affect intracellular viral functions, particularly those that depend on an acidic environment (6, 7).

Our electron microscopy data suggest that VSV is blocked by amantadine, as well as dansylcadaverine, at the stage of viral entry into plasma membrane coated pits. The finding that an agent such as dansylcadaverine, which is known to prevent ligand clustering in coated pits, has a potent antiviral activity may lead to a rational approach to the future identification of nontoxic antiviral compounds. It is too simplistic to suggest that all inhibitors of receptor-mediated endocytosis will also be antiviral agents. Although many viruses (both enveloped and naked) have been observed to enter cells via morphologically identifiable coated pits, there are others that may enter via direct fusion with the plasma membrane. In addition, some inhibitors of α_2M and triiodothyronine internalization (e.g., bacitracin) have no apparent effect upon the entry of VSV (unpublished results). The uptake of a large multivalent particle could differ from the internalization of monovalent ligands, which might require ligand-induced receptor alterations necessary for "clustering" in coated pits.

1. Heine, J. & Schnaitman, C. (1969) *J. Virol.* **3**, 619–622.
2. Heine, J. & Schnaitman, C. (1971) *J. Virol.* **8**, 786–795.
3. Simpson, R., Hauser, R. & Dales, S. (1969) *Virology* **37**, 285–290.
4. Dahlberg, J. (1974) *Virology* **56**, 250–262.
5. Fan, D. & Sefton, B. (1978) *Cell* **15**, 985–992.
6. Marsh, M. & Helenius, A. (1980) *J. Mol. Biol.* **142**, 439–454.
7. Helenius, A., Kartenbeck, J., Simons, K. & Fries, E. (1980) *J. Cell Biol.* **84**, 404–420.
8. Willingham, M., Maxfield, F. & Pastan, I. (1979) *J. Cell Biol.* **82**, 614–625.
9. Dickson, R., Willingham, M. & Pastan, I. (1981) *J. Cell Biol.* **89**, 29–34.
10. Maxfield, F., Willingham, M., Davies, P. & Pastan, I. (1979) *Nature (London)* **277**, 661–663.
11. Cheng, S., Maxfield, F., Robbins, J., Willingham, M. & Pastan, I. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3425–3429.
12. Haigler, H., Willingham, M. & Pastan, I. (1980) *Biochem. Biophys. Res. Commun.* **94**, 630–637.
13. Miller, D., Fever, B., Van Daroef, R. & Lenard, J. (1980) *J. Cell Biol.* **84**, 421–429.
14. Welsh, R., Trowbridge, R., Konalski, J., O'Connell, C. & Pfan, C. (1971) *Virology* **45**, 679–686.
15. Kato, N. & Eggers, H. (1969) *Virology* **37**, 632–641.
16. Dickson, R., Willingham, M. & Pastan, I. (1981) *J. Biol. Chem.* **256**, 3454–3459.
17. Miller, D. & Lenard, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3605–3609.
18. Anderson, P., Tycko, B., Maxfield, F. & Vilček, J. (1982) *Virology* **117**, in press.
19. Davies, W., Grunert, R., Hoff, R., McGahen, J., Neumayer, E., Paulshock, M., Watter, J., Wood, T., Herman, E. & Hoffman, C. (1964) *Science* **144**, 862–863.
20. Maassab, H. & Cochran, K. (1964) *Science* **145**, 1443–1444.
21. Neumayer, E., Hoff, R. & Hoffman, C. (1965) *Proc. Soc. Exp. Biol. Med.* **119**, 393–396.
22. Skehel, J., Hay, A. & Armstrong, J. (1978) *J. Gen. Virol.* **38**, 97–110.
23. De Duve, C., De Barsy, T., Poole, B., Truovet, A., Tulkens, P. & Van Hoff, F. (1974) *Biochem. Pharmacol.* **23**, 4.