Differential Effect of Monensin on Enveloped Viruses that Form at Distinct Plasma Membrane Domains

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ABSTRACT We have observed a striking differential effect of the ionophore, monensin, on replication of influenza virus and vesicular stomatitis virus (VSV) in Madin-Darby canine kidney (MDCK) and baby hamster kidney (BHK21) cells. In MDCK cells, influenza virus is assembled at the apical surfaces, whereas VSV particles bud from the basolateral membranes; no such polarity of maturation is exhibited in BHK21 cells. A 10^{-6} M concentration of monensin reduces VSV yields in MDCK cells by >90% as compared with controls, whereas influenza virus yields are unaffected. In BHK21 cells, monensin also inhibits VSV production, but influenza virus is also sensitive to the ionophore. Immunofluorescent staining of fixed and unfixed MDCK monolayers indicates that VSV glycoproteins are synthesized in the presence of monensin, but their appearance on the plasma membrane is blocked. Electron micrographs of VSV-infected MDCK cells treated with monensin show VSV particles aggregated within dilated cytoplasmic vesicles. Monensin-treated influenza virus-infected MDCK cells also contain dilated cytoplasmic vesicles, but virus particles were not found in these structures, and numerous influenza virions were observed budding at the cell surface. These results indicate that influenza virus glycoprotein transport is not blocked by monensin treatment, whereas there is a block in transport of VSV G protein. Thus it appears that at least two distinct pathways of transport of glycoproteins to the plasma membrane exist in MDCK cells, and only one of them is blocked by monensin.

Ionophores that have an affinity for monovalent cations were previously shown to block the secretion of proteins and glycoproteins in a variety of eukaryotic cells (6, 16, 17, 19). Inhibition of the release of procollagen and fibronectin from human fibroblasts by monovalent ionophores has been reported (6, 19), as well as an arrest in plasma cell immunoglobulin secretion (17). The inhibition of secretion by monensin, a linear polyether with a high affinity for Na^+ ions (9), is accompanied by a pronounced morphological change in the Golgi apparatus, with accumulation of dilated cytoplasmic vacuoles (16, 19). It has been proposed that monensin affects intracellular transport at the level of the Golgi complex by blocking the release of secretory vesicles from Golgi membranes that become dilated as a result of the drug (17).

Cells of the Madin-Darby canine kidney (MDCK) line form monolayers with features of normal epithelia, including a spontaneous electrical potential as well as a net water flux from apical to basal surfaces (1, 8, 10). The cells exhibit junctional complexes that delineate two distinct membrane domains, the apical surfaces characterized by microvilli, and the basolateral surfaces that relate to the basal lamina and to other adjacent cells (1, 12). It has been reported that influenza virus buds exclusively at free apical surfaces of MDCK cells, whereas vesicular stomatitis virus (VSV) matures only at the basolateral membranes (12). This polarity of virus maturation does not require glycosylation of viral glycoproteins (14) but it is likely that the site of insertion of viral glycoproteins into the plasma membrane determines the virus maturation site, because viral glycoproteins are localized in the same membrane domain where budding occurs (11). These findings suggested that distinct pathways for transport of membrane glycoproteins may exist in MDCK cells. Monensin was recently found to block the transport of glycoproteins of VSV and Sindbis virus to the plasma membrane in chicken embryo fibroblast and baby hamster kidney (BHK21) cells (4, 5). Because of the previous results indicating that different pathways are involved in transport of VSV and influenza virus glycoproteins to cell surfaces, we sought to determine whether the replication of influenza virus and VSV can be differentially affected by monensin.

MATERIALS AND METHODS

Materials

Newborn calf serum was obtained from Biocell Laboratories, Carson, Calif. Monensin was purchased from Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.

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Cell and Virus Growth

MDCK and Madin-Darby bovine kidney (MDBK) cells were grown as previously described (13). MDCK cells were maintained in medium supplemented with 5% newborn calf serum. BHK21 cells were grown by described procedures (3). All cells were grown at 37°C in an atmosphere of 5% CO_2 in air.

Stocks of the A/WSN (H_0N_1) strain of influenza virus were prepared in MDBK cells (2), and VSV stocks were grown in BHK21 cells (13). Influenza virus infectivity titers were assayed by plaquing on MDCK cells as previously described (13), except that monolayers were grown on 22.6-mm 12-well culture plates and were inoculated with 0.1 ml of virus dilutions. Infectivity titers of VSV were measured by plaquing on BHK21 cells (13).

Treatment with Monensin

Stock solutions of monensin were prepared in absolute ethanol. Confluent MDCK and BHK21 cells in 60-mm dishes were washed twice with phosphate-buffered saline (PBS) and infected with 10 plaque-forming units (pfu) per cell of either influenza virus or VSV. The adsorption period for influenza virus was 2 h at 37°C, whereas VSV adsorption was 1 h at room temperature. Various concentrations of monensin in serum-free Dulbecco's modified Eagle's medium were added postadsorption. For controls, equivalent concentrations of absolute ethanol were added. Virus yields were determined at 24-h postinfection by plaque assays.

Immunofluorescence

Rhodamine-conjugated rabbit anti-VSV G protein IgG was a gift from M. Roth, and was prepared as described by Winchester (20). VSV-infected MDCK cells (multiplicity of infection [MOI] = 1) grown on cover slips were treated postadsorption with 10^{-5} M monensin as described above. For examination of surface antigens, cells were washed with PBS and treated with 30 mM EGTA in Ca^{2+}-free Eagle's minimal essential medium for 25 min at 37°C. When most cells had rounded up, monolayers were extensively washed with PBS containing 1% bovine serum albumin and 0.03% sodium azide (PBS-BSA), and rhodamine-tagged antiserum was added for 1 h at room temperature. The cells were then washed thoroughly before mounting on glass microscope slides in a 1:1 solution of glycerol and PBS-BSA. For examination of intracellular antigens, cover slips of cells were fixed in 95% ethanol/5% acetic acid for 20 min at −20°C, and stored overnight in PBS-BSA at 4°C before staining for 30 min at room temperature as above. Cells were viewed with an American Optical microscope and photographed with Kodak Ektachrome 200 daylight film.

Electron Microscopy

MDCK cells grown in 60-mm dishes were infected with a multiplicity of 10 pfu/cell for influenza viruses or 50 pfu/cell for VSV, and treated with monensin as described above. At intervals postinfection, cells were washed three times with PBS and fixed *in situ* overnight with 1% glutaraldehyde in PBS. Monolayers were postfixed with 2% osmium tetroxide for 30 min, and dehydrated with 70, 95, and 100% ethanol. The cells were removed as a sheet from their plastic substrate by addition of propylene oxide, and embedded in an epoxy resin mixture. Thin sections were cut on a Sorvall MT2B ultramicrotome (DuPont Company, Biomedical Products Div., Wilmington, Del.), mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 301 electron microscope.

RESULTS

A striking difference was found between the effect of monensin on the yields of infectious influenza viruses and VSV from infected MDCK cells. As shown in Fig. 1, the infectivity titers of VSV were markedly decreased with increasing concentrations of monensin. At a 10^{-6} M monensin concentration, the yield of VSV plaque-forming units was reduced by >90%, and a reduction of $>10^4$ in infectious virus yield was obtained with the highest concentration of monensin tested (10^{-4} M). In contrast, monensin had little or no effect on influenza virus yields in parellel experiments. At a 10^{-6} M concentration of monensin, the virus titer obtained was similar to that from the control cells. Even at a 10^{-4} M monensin concentration, a less than threefold decrease in yield of infectious influenza virus was obtained.

To determine whether virus replication was differentially affected in another cell line that does not exhibit polarity of virus maturation, we examined the effects of monensin on yields of VSV and influenza virus from BHK21 fibroblasts. As seen in Fig. 2, replication of VSV was again found to be very sensitive to the presence of the ionophore. VSV yields were reduced by >99% in the presence of 10^{-5} M monensin, and a reduction of $\sim 10^5$ was observed in the VSV yield in the

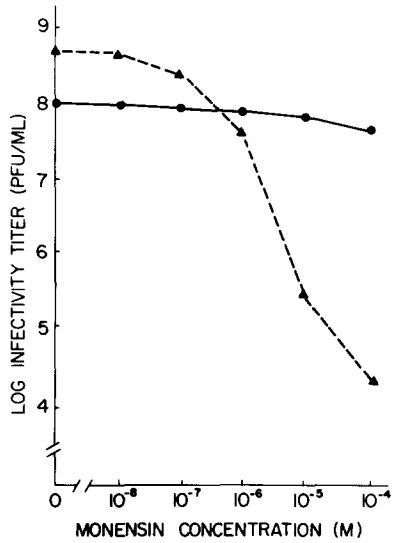

FIGURE 1 Effect of monensin on the yields of infectious influenza virus and VSV in MDCK cells. Monensin was added postadsorption to confluent MDCK monolayers infected with 10 plaque-forming units (pfu) per cell of influenza virus or VSV. Culture fluids were harvested 24 h postinfection. Infectivity titers were determined by plaque assays on MDCK cell for influenza virus and on BHK21 cells for VSV. Influenza virus titers (●), VSV infectivity titers (▲).

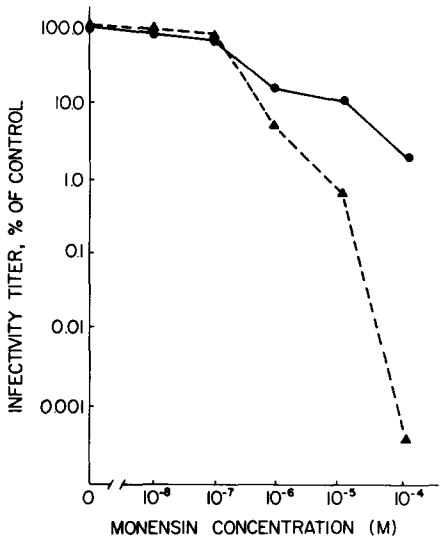

FIGURE 2 Effect of monensin on yields of influenza virus and VSV from BHK21 cells. Confluent BHK21 cell monolayers were infected with 10 pfu per cell of influenza virus or VSV, and monensin was added postadsorption. After 24 h, virus yields were measured by plaque assays. Because influenza virus infectivity titers are usually much lower than that of VSV from BHK21 cells, the results are plotted as percent of control titers to facilitate comparison. Influenza virus titers (●), VSV infectivity titers (▲). At the 100% point, the influenza virus infectivity titer was 9.2×10^7 pfu/ml and the VSV titer was 1.0×10^{10} pfu/ml.

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presence of 10^{-4} M monensin. Influenza virus yields were also reduced significantly in this cell line, particularly when high concentrations of the ionophore were used. It is possible that the ionophore has toxic effects on this cell line in the higher concentration ranges used.

The preceding data, together with previous observations (4, 17, 19), suggest that monensin blocks the transport of VSV glycoproteins to plasma membranes in MDCK and BHK21 cells. We used immunofluorescence to determine whether the VSV glycoprotein is present at the surface of monensin-treated cells. At 9 h postinfection, VSV-infected MDCK cells incubated in the presence or absence of monensin were stained with rhodamine-conjugated anti-VSV G protein IgG. Monolayers of both treated and untreated cells made permeable to antibodies by alcohol–acetic acid fixation showed bright fluorescence in the perinuclear area (Fig. 3). Whereas areas along the plasma membrane of untreated cells also displayed some fluorescence, this was not evident in the presence of monensin. For detection of surface antigens, EGTA was added to unfixed monolayers before the antibody conjugate; this treatment causes cells to round up and thus enables the antibodies to reach the basolateral surfaces of MDCK cells, where the assembly of VSV particles occurs. A brilliant fluorescence was observed along the plasma membrane of cells not treated with monensin (Fig. 3 *C*), whereas monensin-treated cells showed no fluorescence above background levels (Fig. 3 *D*). These results indicate that VSV G protein is synthesized but is not transported to the cell surface in the presence of monensin. Influenza viral antigens were readily observed, however, on surfaces of monensin-treated cells (not shown). In other experiments, we have also demonstrated the synthesis of all major VSV proteins in monensin-treated cells by analysis using SDS polyacrylamide gel electrophoresis (not shown).

The difference in effect of monensin on replication of VSV and influenza virus was also clearly evident when thin sections of infected cells were examined by electron microscopy. VSV-infected MDCK cells treated with 10^{-5} M monensin contained dilated cytoplasmic vesicles, similar to the structures previously described in other monensin-treated cells (5, 17). In most cells, these vesicles were localized near the basolateral surfaces. In some instances, these cytoplasmic vesicles contained VSV particles and other electron-dense material (Fig. 4). Virions were rarely seen forming at the basolateral membrane.

Numerous dilated cytoplasmic vesicles were also observed

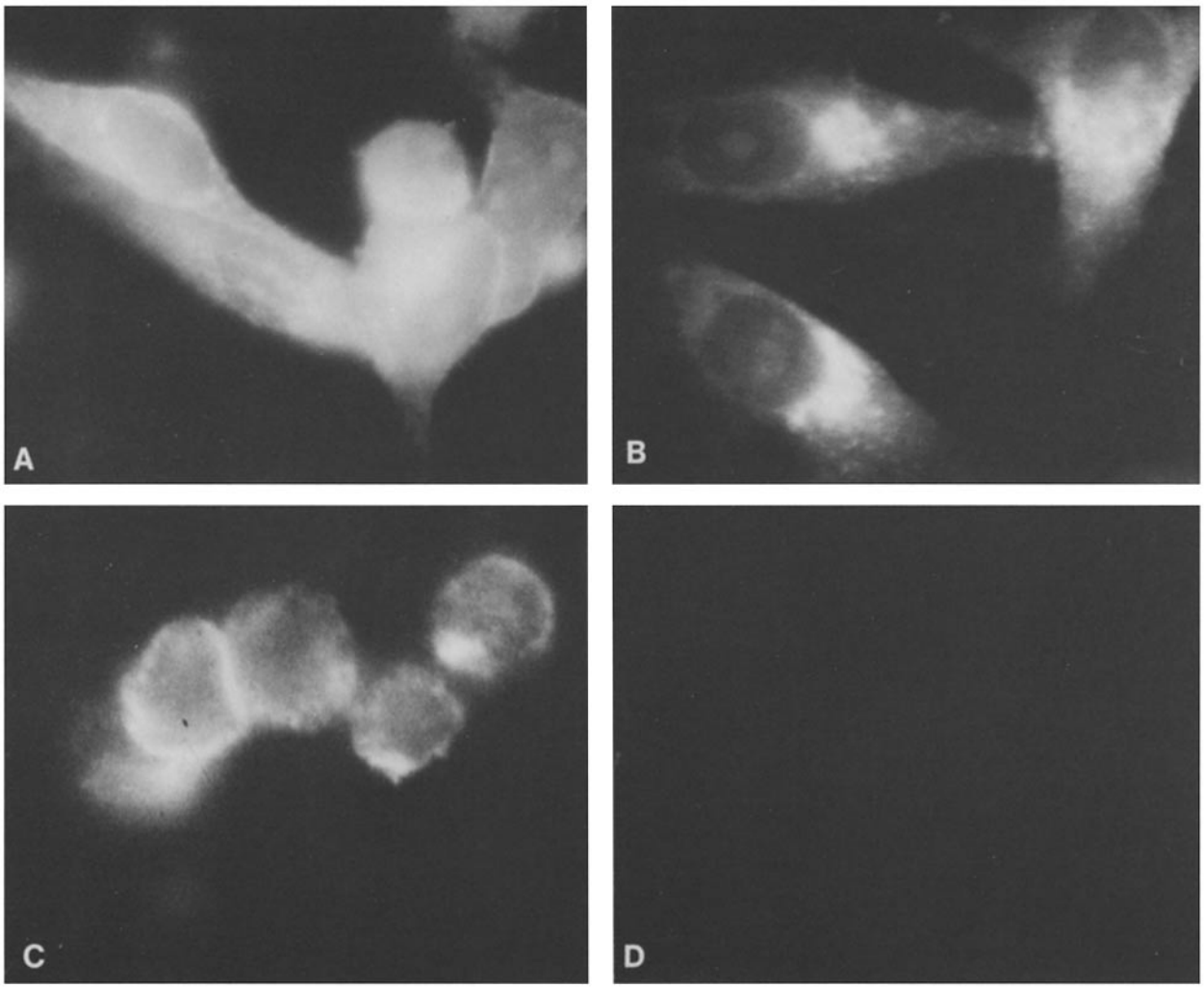

FIGURE 3 Localization of VSV G protein in infected MDCK cells. Cells were infected with VSV and examined by immunofluorescence at 9 h postinfection as described in Materials and Methods. For localization of intracellular antigen, cells were made permeable by alcohol–acetic acid fixation. (*A*) Fixed cells, not treated with monensin. (*B*) Fixed cells, treated with 10^{-5} M monensin. (*C*) Unfixed cells, not treated with monensin. (*D*) Unfixed cells, treated with monensin. × 1,100.

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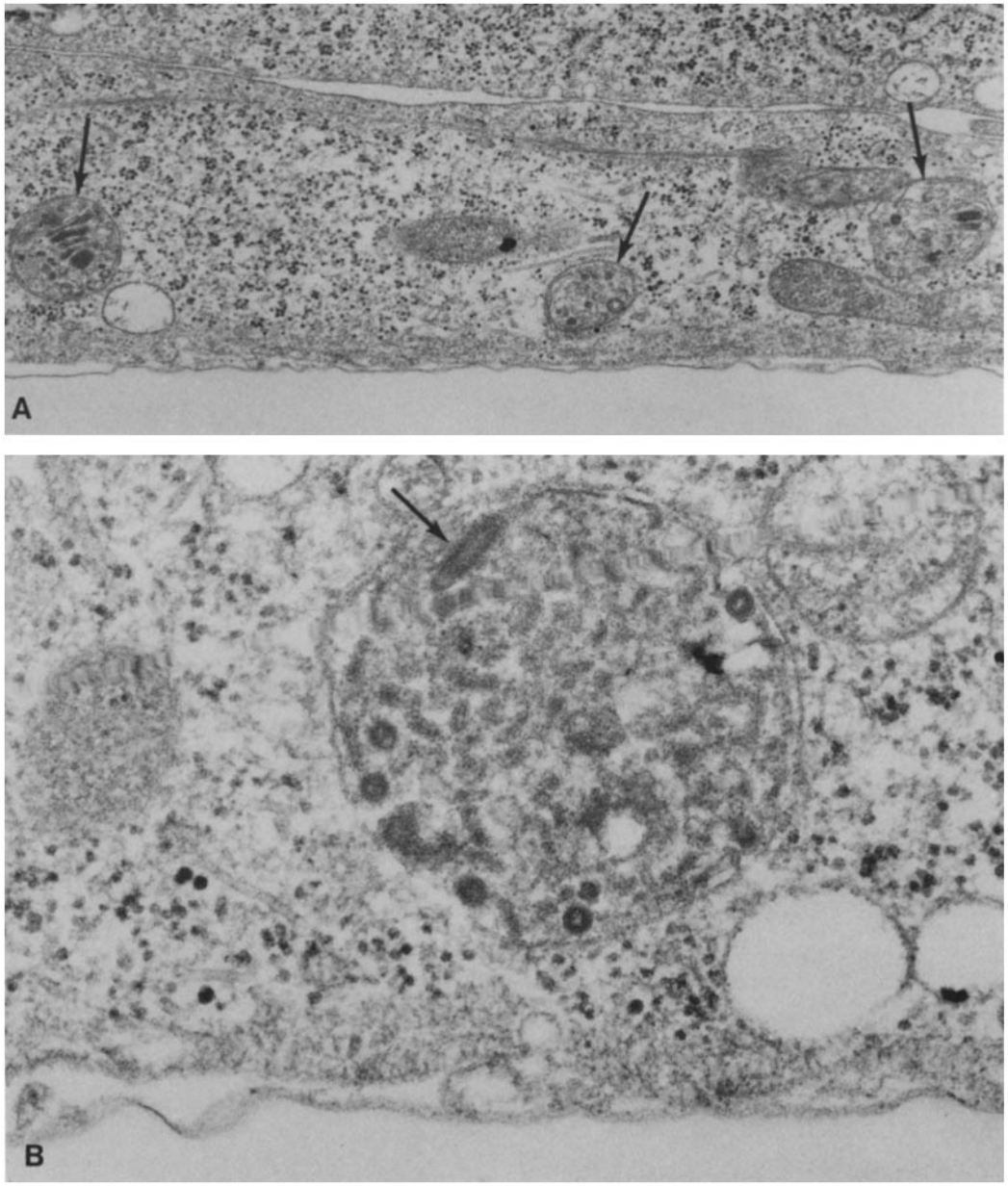

FIGURE 4 VSV-infected MDCK monolayers treated with 10^{-5} M monensin (MOI = 50). (*A*) Low magnification view of a cell at 9 h postinfection showing cytoplasmic vesicles containing viral components (arrows). The basolateral membranes are free of budding VSV virions. × 27,000. (*B*) A cytoplasmic vesicle shown at high magnification containing numerous VSV particles and some additional electron-dense material. A particle lying longitudinally is indicated by an arrow. × 75,000.

in influenza virus–infected MDCK cells treated with 10^{-5} M monensin (Fig. 5). However, no influenza virions were observed within these structures. Numerous influenza virions were found budding at the free apical surfaces, as seen in infected MDCK cells not treated with monensin. In some instances, some virus particles were observed at the basolateral membrane as well; however, this was also observed when controls not treated with monensin were examined at similar time-points, indicating that strict polarity of maturation is lost at later times postinfection.

DISCUSSION

The present results demonstrate that the pathways of transport and insertion of influenza virus and VSV glycoproteins into the plasma membrane of MDCK cells differ with respect to monensin sensitivity. Infectivity titers of VSV are markedly reduced upon treatment of VSV-infected cells with monensin, although all viral proteins are synthesized. The G protein, however, does not appear to reach the cell surface, as was also observed by Johnson and Schlesinger (4) in two other cell types. Surprisingly, influenza virus yields in MDCK cells were unaffected or only slightly affected at the same concentrations of monensin. The difference in effect of monensin on replication of influenza virus and VSV was also apparent when infected cells were examined by electron microscopy. In VSV-infected MDCK cells treated with monensin, virus particles were observed within dilated vacuoles. These cytoplasmic structures are very similar to those found in other monensin-

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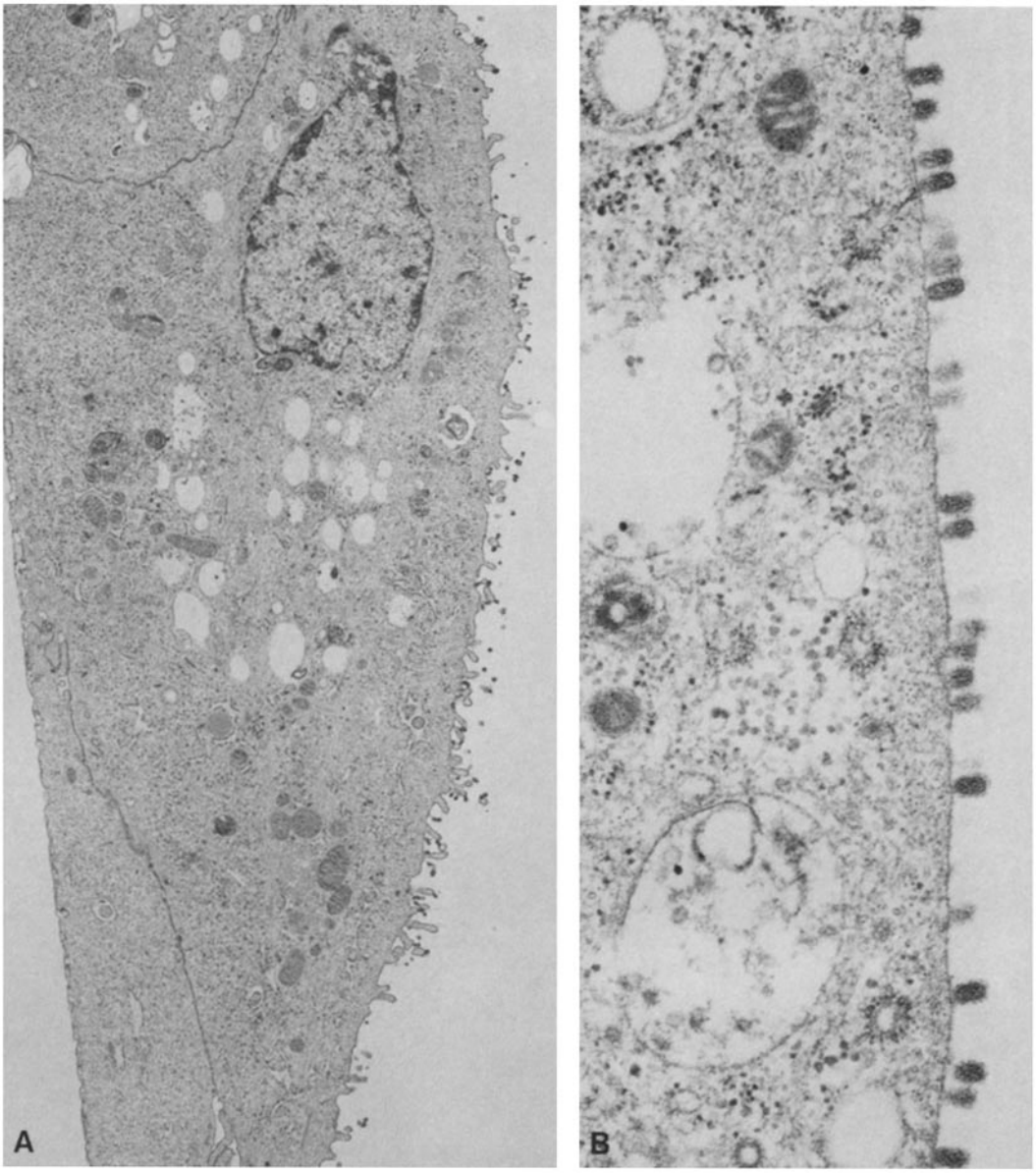

FIGURE 5 Influenza virus-infected MDCK cells treated with 10^{-5} M monensin (MOI = 10). (*A*) An infected cell 12 h postinfection, showing numerous cytoplasmic vesicles that appear empty. Influenza virions are associated with the apical surfaces. × 7,800. (*B*) Higher magnification view of influenza virions budding from the apical membrane in a monensin-treated cell. × 45,000.

treated cells (4, 17). The observed accumulation of VSV particles in vacuoles may be a result of budding of virus into cytoplasmic vesicles, as has been observed with Sindbis virus in the presence of the ionophore (4). In contrast, influenza virus–infected cells treated with the same concentrations of monensin showed numerous virions budding at the free apical surfaces, as in untreated cells. Dilated cytoplasmic membranes were also observed in these cells but no influenza virus particles were found inside these structures. Because the production of infectious influenza virus requires the presence of two functional glycoproteins, hemagglutinin and neuraminidase, at the plasma membrane, we conclude that the transport of influenza virus glycoproteins is insensitive to monensin treatment in MDCK cells, whereas VSV glycoprotein transport to the cell surface is impaired by monensin treatment.

A recent report indicates that newly synthesized VSV glycoproteins are transported from the endoplasmic reticulum to the plasma membrane via clathrin-coated vesicles (15). It is possible that monensin blocks exit of these coated vesicles from the Golgi complex. The ionophores are thought to impede secretion by acting on secretory structures rather than on the proteins themselves (16). There is no information as yet on the composition of structures involved in mediating the transit of influenza virus glycoproteins to the cell surface, but our results suggest that these glycoproteins are transported by vesicles whose formation is insensitive to monensin.

Other viral and cellular membrane proteins also exhibit polarized distributions in epithelial cells (7, 11). It will be of interest to determine whether the differences in response to monensin observed with VSV and influenza viral glycoproteins reflect general properties of proteins localized in apical vs. basolateral membrane domains. The use of ionophores that selectively block transport of one class of membrane proteins should also aid in elucidating the mechanisms by which membrane glycoproteins are transported from the Golgi complex to specific cell surface domains.

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