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Monensin inhibits Semliki Forest virus penetration into culture cells

(carboxylic ionophores/virus entry/lysosomotropic agents/lysosomes/cell fractionation)

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ABSTRACT **The carboxylic ionophores monensin and nigericin, at concentrations higher than 10 and 6 μM, respectively, prevent the penetration of the Semliki Forest virus (SFV) genome into the cytosol of baby hamster kidney (BHK-21) cells and thereby inhibit viral replication. In the absence of inhibitors, the entry of SFV is known to proceed by adsorptive endocytosis in coated vesicles, followed by acid-triggered membrane fusion in intracellular vacuoles or lysosomes. The results show that binding of the virus to the cell surface, adsorptive endocytosis, and intracellular transport of viruses to the lysosomes are only marginally affected by the ionophores. No direct virucidal effect is observed, nor is the membrane fusion activity of the virus at low pH directly affected. Sequential addition of monensin and ammonium chloride (a nonrelated lysosomotropic inhibitor of SFV entry) indicates that both inhibitors affect the same step in the entry pathway. On the basis of these data and the known effects of carboxylic ionophores and lysosomotropic weak bases on cellular pH gradients, we conclude that monensin inhibits penetration by increasing the pH in endocytic vacuoles and lysosomes above pH 6, which is the pH threshold for the viral membrane fusion activity.**

Monensin and nigericin are carboxylic ionophores that intercalate into membranes and abolish proton gradients by electroneutral transmembrane exchange of protons for monovalent cations (1). When added to cells, monensin causes a variety of effects depending on cell-type, concentration, and time of incubation. These include inhibition of protein secretion, the transport of plasma membrane glycoproteins to the cell surface (2–4), serum low density lipoprotein uptake and degradation (5), membrane protein recycling (5, 6), and fluid-phase endocytosis (6). It has also been shown that monensin blocks the entry of vesicular stomatitis virus and diphtheria toxin into tissue culture cells (7, 8).

In this study we have examined the effects of monensin and nigericin on the entry of Semliki Forest virus (SFV) into baby hamster kidney (BHK-21) cells. This simple enveloped virus is internalized by an endocytic pathway that involves coated pits, coated vesicles, endosomes, and secondary lysosomes (9, 10). Penetration of the viral genome into the cytosol occurs when the virus reaches an organelle with a pH < 6. The mildly acidic pH induces a potent membrane fusion activity in the virus that results in a fusion reaction between the viral membrane and the vacuolar membrane (11, 12). The nucleocapsids are thereby transferred into the cytoplasmic compartment. Nonfused viruses and viral membrane proteins are subsequently degraded in the lysosomes (10).

Our results indicate that the principal effect of monensin (and possibly nigericin, for which our data is less complete) during virus entry is to inhibit the penetration of the viral nucleocapsid from the intracellular vacuoles into the cytosol.

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MATERIALS AND METHODS

Cells, Virus, and Inhibitors. BHK-21 cells were grown in Glasgow minimum essential medium as described (9). A prototype strain of SFV, [^{35}S]methionine-labeled SFV (^{35}S-SFV), and [^{32}P]orthophosphate-labeled SFV (^{32}P-SFV) were propagated in BHK-21 cells (13, 14). The ^{32}P-SFV had 50% of the radioactivity in the RNA and 50% in the phospholipid. Monensin and nigericin were purchased from Calbiochem. Viral infection was assayed by incorporation of [^{3}H]uridine into acid-precipitable viral RNA as described (15).

Binding, Endocytosis, and Degradation. Binding, endocytosis, and degradation of ^{35}S-SFV was determined as described (10). Proteinase K digestion was used to distinguish between surface-bound and internalized viruses (10).

Cell Fractionation. BHK-21 cells were fractionated by free-flow electrophoresis as described (16). Briefly, after incubation with ^{32}P-SFV for 1.5 hr at 37°C, the cells were harvested by scraping in phosphate-buffered saline and washed twice in lysis buffer (0.25 M sucrose/10 mM triethanolamine/10 mM acetic acid/1 mM EDTA, pH 7.4). The cells were lysed on ice by 20 passes through a 10-ml glass pipette. Intact cells (30%), nuclei, and aggregates were removed by three successive 10-min centrifugations at 750, 850, and 950 × *g*, and the lysate was passed through one layer of prewashed filter paper (Schleicher and Schuell, no. 589/3). The filtrate was incubated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (10 μg ml^{-1}; Worthington) for 3 min at 37°C before addition of soybean trypsin inhibitor (20 μg ml^{-1}; Sigma). The lysates were fractionated by free-flow electrophoresis, and the fractions were assayed for organelle enzyme markers as described (16) and for radioactivity.

Microscopy. For electron microscopy of thin sections, the cells were fixed by using 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2). Dehydration, sectioning, and staining were performed as described (9).

RESULTS

Monensin and Nigericin Inhibit SFV Infectivity. It has been shown (17) that addition of 1 μM monensin to SFV-infected cells inhibits the formation of progeny virus by blocking a late event in the replication cycle. The viral components are synthesized,

Abbreviations: SFV, Semliki Forest virus; BHK, baby hamster kidney; moi, multiplicity of infection; pfu, plaque-forming unit(s); INT, 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride; ^{35}S-SFV, [^{35}S]methionine-labeled SFV; ^{32}P-SFV, [^{32}P]orthophosphate-labeled SFV.

but virus maturation is inhibited because the spike glycoproteins are not transported to the cell surface (17, 18). Thus, to study the effects of monensin and nigericin on early stages of infection, we measured incorporation of [^{3}H]uridine into viral RNA. This assay monitors virus entry without relying on the production of progeny virus (15, 19). We found that, in addition to the effect on virus maturation (17, 18), the ionophores inhibited an early step in the replication cycle prior to initial RNA synthesis. This effect required higher ionophore concentrations. With 10 μM monensin or 3 μM nigericin, incorporation of [^{3}H]uridine into viral RNA was inhibited by $\approx$60% (Fig. 1).

With 1.0 μM monensin, the incorporation of [^{3}H]uridine was not inhibited but reproducibly exceeded that observed in untreated control cells (Fig. 1). This increase in labeling of viral RNA in the presence of low monensin concentrations was consistent with a block in virus maturation (17) because, unlike the control cells, no labeled RNA was expected to be lost from monensin-treated cells in the form of mature virus. This interpretation was supported by experiments in which 1.0 μM monensin was added 3 hr after infection instead of 20 min prior to infection. The same increase in incorporation was observed, indicating that, when maturation is inhibited, more labeled viral RNA remains associated with the cell. Most of the subsequent studies used 10 μM monensin and, to prevent discrepancies arising from loss of radioactivity through virus maturation, the inhibitor was included in all samples during the period of [^{3}H]uridine labeling.

The efficiency of inhibition depended on the time of monensin addition. No inhibition was detected when 10 μM monensin was added simultaneously with the SFV inoculum, whereas 60 min of prior incubation gave 85% inhibition. With 25 μM monensin, a 5-min preincubation was sufficient for full inhibition, whereas 1.0 μM monensin was without effect after

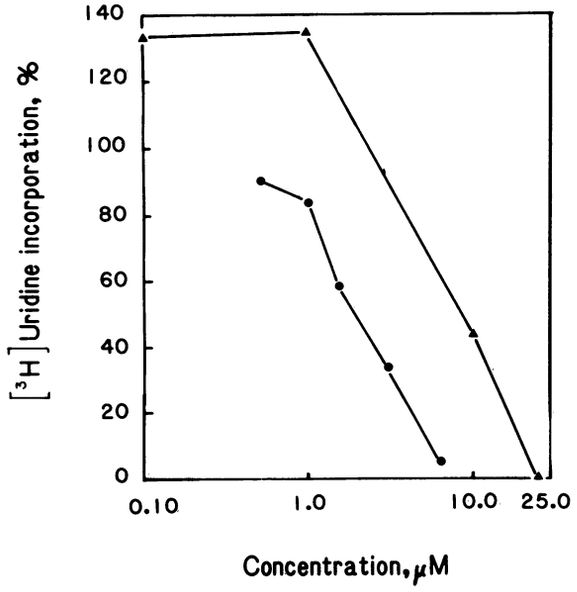

FIG. 1. Effect of monensin and nigericin on viral RNA-synthesis. Cells were preincubated at 37°C with medium containing monensin (▲) or nigericin (●). The medium was removed after 20 min, and the cells were infected with SFV [20 plaque-forming units (pfu)/cell] for 90 min. Subsequently, the cells were incubated for 30 min in medium containing actinomycin D (2 μg ml^{-1}) and finally in medium containing actinomycin D and [^{3}H]uridine. Monensin or nigericin was present throughout. The cells were washed and precipitated with trichloroacetic acid, and the [^{3}H]uridine incorporation was determined. Incorporation is expressed as a percentage of the [^{3}H]uridine incorporation into SFV-infected cells in the absence of ionophore (100% is equivalent to 15,000 cpm).

120 min of preincubation. The lack of inhibition with 10 μM monensin and SFV added simultaneously to cells indicated that monensin had no direct virucidal effect. Apparently all of the viral functions in entry were intact in spite of the presence of monensin. When 25 μM monensin was added 2.5 hr after infection (i.e., when virus entry had already taken place), [^{3}H]uridine incorporation into viral RNA was unaffected. Similarly, monensin (25 μM) had no effect on [^{3}H]uridine incorporation into cellular RNA in the absence of virus. These results indicate that monensin blocks a step in the virus entry pathway prior to the onset of RNA replication and that it does not directly affect the functions of the virus particle or the RNA replication process.

Bypass of the Monensin Block. SFV penetration into BHK-21 cells is inhibited by lysosomotropic weak bases, this inhibition can be bypassed by artificially induced fusion of the viruses with the plasma membrane at pH 5.5 (9, 12, 15). To see whether the monensin block could be similarly bypassed, SFV in the presence of 10 μM monensin was bound to duplicate wells of monensin-treated cells. To induce fusion, the medium in one well was replaced with pH 5.5 medium (containing monensin) for 1 min. The other well served as a control and was kept at pH 7.0 throughout. The cells were then incubated for 2 hr in the presence of monensin and assayed for viral RNA synthesis. Table 1 shows that the cells treated at pH 5.5 were infected in the continuous presence of monensin, whereas cells kept at neutral pH throughout were inhibited.

This result confirms that monensin has no direct virucidal effect and does not affect the low pH-dependent fusion activity of the virus as such. In addition, it demonstrates that the viral genome, when introduced into the cytosol in the presence of monensin, is fully capable of uncoating and serving as messenger for initial protein synthesis and as a template for RNA replication.

In view of these findings, the most likely explanation for the early inhibitory effect of monensin is that the viral RNA does not reach the cytoplasmic matrix. To test this possibility directly, we relied on the sensitivity to RNase of uncoated nucleocapsids (15). We determined the fraction of parental [^{32}P]RNA accessible to RNase in isotonic lysates of cells that had been incubated prior to lysis with ^{32}P-SFV for 90 min at 37°C. In control cells, 40% of the cell-associated viral [^{32}P]RNA was accessible to digestion by the RNase. In cells treated with 10 μM monensin, <1% of the viral RNA was accessible. This indicated that the nucleocapsids were not released from the protective viral envelope and transferred into the cytosol.

Effect on Binding and Internalization. As the delivery of nucleocapsids into the cytosol is blocked by monensin, we used biochemical and morphological techniques to determine at which stage in the entry pathway the block occurred. For the morphological studies, SFV was added to cells for 60 min at 0°C, after which the unbound viruses were washed away and the cells were warmed to 37°C. Indirect immunofluorescence indicated

Table 1. Low pH-induced bypass of the monensin block

Inhibitor	pH 5.5 treatment	Incorporation, cpm × 10^{-4}
None	−	3.3
None	+	4.7
Monensin, 10 μM	−	0.1
Monensin, 10 μM	+	2.8

A virus inoculum of 60 pfu/cell was allowed to bind for 10 min at 0°C. Where indicated, monensin was present in all media and was included in all samples during [^{3}H]uridine labeling. A background (noninfected cells) of 0.5 × 10^4 cpm was deducted from all values.

that, both in control cells and in treated (10 μM monensin or 3 μM nigericin) cells, the viral antigens were almost totally cleared from the cell surfaces within 1 hr and were relocated into intracellular vacuoles (not shown).

Transmission electron microscopy of thin sections also indicated that normal uptake occurred in the presence of 20 μM monensin or 3 μM nigericin. Viruses were seen in coated pits, coated vesicles, and in large (500 μm) and small uncoated (150–200 μm) cytoplasmic vacuoles (Fig. 2). The vacuoles, some containing only a few virus particles and others packed with many viruses, were similar in control cells and in monensin- and nigericin-treated cells.

The quantitative studies with ^{35}S-SFV at low (15 particles per cell) and high (2.3×10^4 particles per cell) multiplicity of infection (moi) showed that binding to 10 μM monensin-treated cells in the cold was 5–10% lower than to control cells, and the rate of uptake of the prebound viruses upon warming was lower by 20% (at low moi; see Fig. 3) to 35% (at high moi). The rate of internalization was also decreased by 30% when SFV (9,000 particles per cell) was added to cells at 37°C without prior binding. Increasing the monensin concentration to 25 μM did not decrease the rate of uptake further. To determine whether the decrease in internalization rates was restricted to virus uptake or whether it reflected a general decrease of cellular pinocytic activity, we measured the uptake of two fluid phase markers, horseradish peroxidase (20) and [^{3}H]sucrose (10) in the presence and absence of 10 μM monensin. A 50% reduction was observed in the accumulation of both markers (controls accumulated the equivalent of 0.44 μl/hr per 10^7 cells) which suggested that fluid pinocytosis was also depressed by the drug. However, the inhibition observed in virus uptake (20–35%) is too small to account for the almost total inhibition of infection. Thus, the primary block to infection cannot be ascribed to inhibition of binding and internalization but must occur after the viruses have undergone endocytosis.

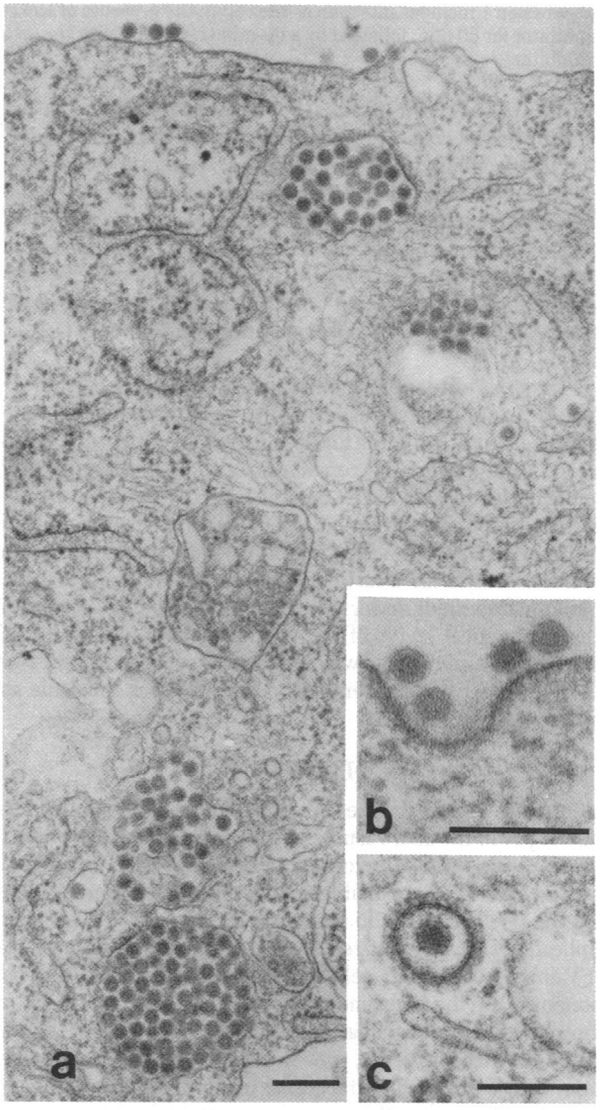

FIG. 2. Entry of SFV into BHK-21 cells in the presence of monensin. SFV was bound to BHK-21 cells (60 min at 0°C) in the presence of 2 μM monensin. The cells were washed and then warmed to 37°C in medium containing 20 μM monensin for 20 min. The cells were fixed and processed for electron microscopy. (*a*) Viruses in small and large cytoplasmic vesicles. (×44,000.) (*b*) Viruses associated with coated-pit regions of the cell surface. (×100,000.) (*c*) Viruses in coated vesicles. (×76,000.) (Bar = 0.2 μm.) Similar figures were seen in 3 μm nigericin-treated cells (not shown).

Passage of SFV into Lysosomes. The most dramatic difference between the monensin-treated and control cells shown by the biochemical uptake assays was the almost total inhibition of virus protein degradation (Fig. 3). Whereas 25–35% of the [^{35}S]methionine in control cells became acid soluble within 90 min at 37°C, <5% was degraded in the monensin-treated cells. To determine whether the viruses were transported into the lysosomal compartment in the presence of monensin (10 μM), cells were exposed to ^{32}P-SFV for 90 min and lysed under isotonic conditions, and the postnuclear supernatant was fractionated by free-flow electrophoresis. With cultured human skin fibroblasts, two prominent peaks of enzyme activities are routinely obtained with the technique (16). The first, migrating furthest toward the anode, contains marker enzymes for lysosomes—β-*N*-acetylglucosaminidase and acid β-glycerophosphatase. The second peak contains the majority of the protein and marker enzymes for other membrane organelles—e.g., mitochondria [succinate:3-(4-iodophenyl)-2-(4-nitrophenyl)-5-

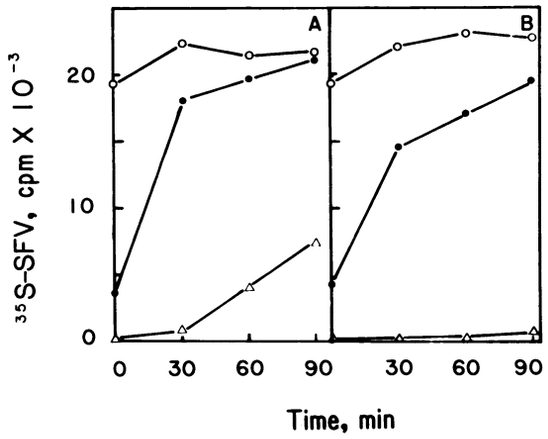

FIG. 3. The influence of monensin on the binding, endocytosis and degradation of SFV with BHK-21 cells. Cells were pretreated with binding medium with or without 10 μM monensin for 60 min at 37°C. ^{35}S-SFV (15 particles per cell) was bound to cells in the presence (*B*) or absence (*A*) of monensin (10 μM) for 1 hr in binding medium at 0°C. The unbound viruses were washed away, and the cells were warmed to 37°C in binding medium (with or without monensin). At given time points, the cells were assayed for total cell-associated activity and for proteinase K-resistant activity, and the medium was assayed for degraded activity as described. ○, Total cell-associated activity, including degraded (medium trichloroacetic acid soluble) activity; ●, total internalized activity (i.e., proteinase K-resistant plus degraded activity); △, degraded activity.

phenyl-2H-tetrazolium chloride (INT) oxidoreductase (EC 1.3.99.1)], endoplasmic reticulum (NADPH-cytochrome *c* reductase), plasmalemma (Na^+K^+-ATPase), and Golgi (UDPgalactose–glycoprotein galactosyltransferase) (16). With a BHK-21 cell lysate, a similar separation was observed (Fig. 4 *Upper*). The fractions containing lysosomes showed 22-fold enrichment of β-*N*-acetylglucosaminidase over the initial lysate and in excess of 90% latency of this enzyme. Thin-section electron microscopy of a pellet derived from the lysosomal fractions showed a relatively homogeneous population of secondary lysosomes.

Analysis of the ^{32}P activity associated with the fractions indicated that ≈30% of the viral activity cofractionated with the lysosomes in monensin-treated cells (Fig. 4), NH_4Cl-treated cells, and control cells. The results suggest that monensin and NH_4Cl do not prevent virus transport into lysosomes; however, we have no reliable markers for endosomes that may contaminate the lysosomal fractions. Whether the viral ^{32}P activity that is not in the lysosomal fraction is associated with endosomes remains to be determined.

Relationship with NH_4Cl-Induced Inhibition. Lysosomotropic weak bases, such as NH_4Cl, inhibit SFV infection by elevating the pH in intracellular vacuoles to values higher than that needed to trigger the fusion reaction (15, 21, 22). To test whether inhibition by monensin and NH_4Cl occur at the same stage of virus entry, we adopted a strategy used by Marnell *et al.* to study diphtheria toxin penetration (8). These experiments rely on the reversibility of both inhibitors and on the assumption that they affect single steps in the pathway. If inhibitor I acts prior to inhibitor II, incubation of cells and viruses with I and subsequently with II should not lead to infection; however, incubation in the reverse order should permit infection. If I and II affect the same step, no infection should occur regardless of the order in which the inhibitors are applied.

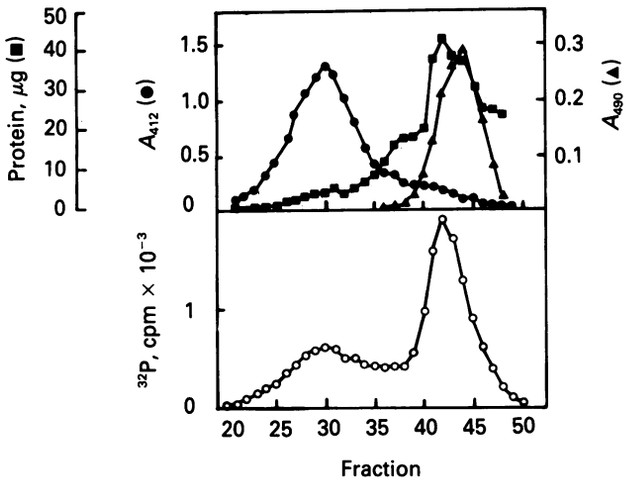

FIG. 4. Fractionation of BHK-21 cells infected with ^{32}P-SFV in the presence of monensin. Cells, on 850-cm^2 roller bottles, were pretreated with binding medium containing 10 μM monensin for 30 min at 37°C. ^{32}P-SFV in this medium with monensin was incubated with the cells for 60 min at 37°C, the unbound virus was washed away, and the cells were incubated for a further 30 min in virus-free binding medium with monensin at 37°C. Subsequently the cells were harvested, lysed, and fractionated by free-flow electrophoresis (16). Each fraction was assayed for total protein content (■), for β-*N*-acetylglucosaminidase by measurement of A_{412} (●), for succinate:INT oxidoreductase by measurement of A_{490} (△) as described (16), and for ^{32}P activity (○). Of the initial cell-associated ^{32}P activity, 64% remained for fractionation; of this, 79% was recovered. For cell protein, 50% was fractionated and 74% was recovered; for succinate:INT oxidoreductase, 52% was fractionated and 80% was recovered; and for β-*N*-acetylglucosaminidase, 67% was fractionated and 99% was recovered.

Table 2. Concurrence of monensin and NH_4Cl inhibition

Exp.	Incubation 1 (60 min)	Incubation 2 (120 min)	^3H, cpm × 10^{-4}
Control	None	None	15.5 (100%)
	Mon	Mon	0
	NH_4Cl	NH_4Cl	0
Reversibility	Mon	None	7.5 (48%)
	NH_4Cl	None	9.9 (64%)
Substitution	Mon	NH_4Cl	0
	NH_4Cl	Mon	0
Substitution and reversibility*	Mon, NH_4Cl	None	5.6 (36%)
	NH_4Cl, Mon	None	5.9 (38%)

Mon, monensin. The monensin was 10 μM, NH_4Cl was 15 mM, and the SFV inoculum was 20 pfu/cell. [^3H]uridine and 10 μM monensin were added to all samples for 2 hr after incubation 2. Figures in brackets express the percentage of the uninhibited control.

* Incubation 1 includes infection of the cell in the presence of the first inhibitor for 60 min, followed by a 60-min treatment with the second inhibitor.

First we demonstrated that monensin inhibition is reversible. Pretreated cells were incubated with SFV in the presence of 10 μM monensin for 60 min at 37°C. Monensin-free medium was added, and the cells were incubated for a further 2 hr before [^3H]uridine labeling. We measured 50% infection compared to that in inhibitor-free control cells (Table 2). Cells treated with monensin throughout were fully inhibited. The reversibility of NH_4Cl inhibition has been demonstrated (15); in the present case, we recorded 60% infection after removal of NH_4Cl (15 mM).

Two types of double inhibition experiments are shown in Table 2. In substitution experiments, cells were infected in the presence of one inhibitor for 60 min and then treated with the second inhibitor for the remainder of the experiment. No labeling occurred with either combination of inhibitors. Second, in substitution and reversion experiments, cells were infected in the presence of the first inhibitor for 60 min, then treated with the second for a further 60 min, and finally returned to inhibitor-free medium for 2 hr before addition of [^3H]uridine. Almost identical levels of incorporation (36% and 38%) were observed irrespective of which inhibitor was used first. These results suggest that monensin and NH_4Cl block the same step in the infection process.

DISCUSSION

Lysosomotropic weak bases inhibit SFV penetration by increasing the pH in the acid vacuoles of the endocytic pathway (9, 15, 21, 22). When the pH exceeds a value of 6, the viruses endocytosed by the cell are unable to fuse with vacuolar membranes, and the nucleocapsids do not reach the cytoplasmic matrix for replication (12, 15). Our results suggest that monensin inhibits SFV entry into BHK-21 cells by a similar mechanism. With both monensin and the lysosomotropic weak bases (9, 10, 12, 15), we observe no direct virucidal effect and no inhibition of the viral membrane fusion activity, provided that the pH is properly lowered. Endocytosis and intracellular transport appear to function normally, although with monensin a partial inhibition of overall cellular pinocytic activity and virus uptake was observed. The block to virus entry imposed by both types of inhibitor can be circumvented readily by low pH-induced virus fusion at the plasma membrane (12), and substitution experiments indicate that monensin and NH_4Cl inhibit entry at the same stage. The conclusion that the main inhibitory effect involves a failure to trigger the low pH-dependent fusion in intracellular vacuoles and lysosomes is consistent with the observation of Ohkuma and

Poole (21) that both types of agent increase the lysosomal pH. Carboxylic ionophores collapse the proton gradient by transmembrane exchange of protons for monovalent cations (1), and the lysosomotropic weak bases act as proton traps (see ref. 2).

These results are quite similar to those of Marnell *et al.* (8), who have studied the inhibition of diphtheria toxin entry by monensin. The toxin enters cells by endocytosis, followed by a low pH-dependent penetration of the toxic A subunit that can be blocked both by monensin and lysosomotropic weak bases (8, 23, 24). Monensin also has been shown to inhibit infection by vesicular stomatitis virus into Swiss 3T3 mouse cells (7). This enveloped virus infects cells by a mechanism similar to SFV (25), and its entry is sensitive to lysosomotropic weak bases (25–27). However, Schlegel *et al.* (7) attribute the primary inhibition of entry to a decreased rate of virus endocytosis. We also observe a decrease in endocytosis, but the effect is not large enough to account for the comparatively high inhibition of SFV-infection. Whether a true difference in the action of monensin exists between the two virus systems is not clear. However, it is evident that carboxylic ionophores, depending on dosage, time of action, and conditions, may affect a variety of cellular functions (2–6) and, although our results show that the main effect on SFV entry occurs at the level of penetration, other effects may indeed contribute to the overall levels of inhibition.

One of the most striking effects of monensin is the almost complete inhibition of degradation of virus proteins in the cell. Inhibition of degradation of low density lipoprotein in monensin-treated cells has been shown by Basu *et al.* (5). Two possible explanations could account for this effect—either the lysosomal hydrolases are inhibited by the drug or the delivery of virus into the lysosomal compartment is impaired. The fact that similar amounts of viral radioactivity were found in the lysosomal peak after cell fractionation of monensin-treated, ammonium chloride-treated, and control cells suggests that delivery to lysosomes is not impaired. However, these conclusions must remain somewhat tentative, as we do not yet know the endosomal contamination of our lysosome fractions.

It is interesting that 70% of the intracellular viruses are not in lysosomes at 90 min. We have assumed in our previous studies that the acid vacuoles from which penetration occurs are secondary lysosomes. However, our kinetic results suggest that the viruses enter a low pH compartment as early as 2–4 min after leaving the cell surface (9, 28). Thus, the initial acid encounter and the resulting membrane fusion reaction appear to take place very early and may involve prelysosomal vacuoles (endosomes). In fact, the viruses that enter the secondary lysosomes may represent particles that have failed to fuse in the endosomes. The relationship between endosomes and lysosomes in the process of virus entry and endocytosis in general presents an intriguing problem. Carboxylic ionophores may provide a useful tool in the detailed dissection of the pathway.

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