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Rotaviruses Induce an Early Membrane Permeabilization of MA104 Cells and Do Not Require a Low Intracellular Ca^{2+} Concentration To Initiate Their Replication Cycle

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In this work, we found that rotavirus infection induces an early membrane permeabilization of MA104 cells and promotes the coentry of toxins, such as α-sarcin, into the cell. This cell permeability was shown to depend on infectious virus and was also shown to be virus dose dependent, with 10 infectious particles per cell being sufficient to achieve maximum permeability; transient, lasting no more than 15 min after virus entry and probably occurring concomitantly with virus penetration; and specific, since cells that are poorly permissive for rotavirus were not permeabilized. The rotavirus-mediated coentry of toxins was not blocked by the endocytosis inhibitors dansylcadaverine and cytochalasin D or by the vacuolar proton-ATPase inhibitor bafilomycin A1, suggesting that neither endocytocis nor an intraendosomal acidic pH or a proton gradient is required for permeabilization of the cells. Compounds that raise the intracellular concentration of calcium ($[Ca^{2+}]_i$) by different mechanisms, such as the calcium ionophores A23187 and ionomycin and the endoplasmic reticulum calcium-ATPase inhibitor thapsigargin, did not block the coentry of α-sarcin or affect the onset of viral protein synthesis, suggesting that a low $[Ca^{2+}]_i$ is not essential for the initial steps of the virus life cycle. Since the entry of α-sarcin correlates with virus penetration in all parameters tested, the assay for permeabilization to toxins might be a useful tool for studying and characterizing the route of entry and the mechanism used by rotaviruses to traverse the cell membrane and initiate a productive replication cycle.

Rotaviruses, members of the family *Reoviridae*, are nonenveloped viruses that posses a genome of 11 segments of double-stranded RNA contained in a triple-layer protein capsid (44). The outermost layer is composed of two proteins, VP4 and VP7. The smooth external surface of the virus is made up of 780 copies of glycoprotein VP7, while 60 spikelike structures, formed by dimers of VP4, extend about 12 nm from the VP7 surface (2, 38).

VP4 has essential functions in the virus life cycle, including receptor binding and cell penetration (15). The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity. In addition, the infectivity of rotaviruses is increased by and most probably is dependent on trypsin treatment of the virus, and this proteolytic treatment results in the specific cleavage of VP4 to polypeptides VP8 and VP5 (14, 16). The cleavage of VP4 does not affect cell binding (9, 20, 27) and has been associated with the entry of the virus by direct cell membrane penetration (27, 35, 41). The role of VP7 during the early interactions of the virus with the cell is not clear, although it has been shown that it can modulate some of the VP4-mediated virus phenotypes, including receptor binding (33).

Rotavirus infection is highly restricted in vivo to the mature villus tip cells of the small intestine. In vitro infection is also restricted, being most permissive in a variety of epithelial cell lines of renal and intestinal origins (15). Most animal rotaviruses bind to the cell surface through a sialic acid-containing cell receptor (15). This interaction, however, is nonspecific and can be superseded by an interaction with a secondary receptor which has been proposed to be more specific and may explain, at least in part, the tropism of these viruses (5, 32).

The mechanism by which rotaviruses enter the host cell is still a matter of debate. Most available data are compatible with the virus entering the cell by direct penetration at the plasma membrane level; however, these data do not completely rule out the possible entrance of the virus by endocytosis (20, 46). Both mechanisms appear to take place, but it has been proposed that the entry of the virus through the endocytic route leads to a nonproductive infection (4, 20, 46, 47). This proposal is based on the fact that treatment of cells with lysosomotropic agents, which raise the endosomal pH, or with drugs that block endocytosis or the intracellular transit of endocytic vesicles (4, 21, 27, 31) does not affect the infectivity of rotaviruses. Additional support is provided by the observation that uncleaved, nonactivated virus is preferentially taken up by the endocytic route (27, 46).

After the virus enters the cell, viral transcriptase is activated and the synthesis of viral mRNAs is initiated. In vitro treatment of complete viral particles with chelating agents results in the removal of the outer-layer proteins, VP4 and VP7, leading to transcriptionally active double-layer particles (10, 45). It has been suggested that in vivo the penetration of virions into the cell cytoplasm, which has a Ca^{2+} concentration several orders of magnitude lower than that in the extracellular medium, may be the factor that triggers the uncoating of the virus and activates viral transcriptase (10, 31). Little is known, however, about the mechanism by which rotaviruses uncoat during the infection process.

It has been shown for many different viruses that the entry of viral particles into the cell interior induces an early membrane

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permeabilization of the cell, allowing the coentry of macromolecules, such as toxins (7, 18). The mechanism by which this coentry occurs is still not well understood; however, it seems to be a rather general phenomenon that takes place irrespective of the route of entry of the virus. Thus, enveloped viruses that enter via receptor-mediated endocytosis or enveloped viruses that enter the cell by fusion of the envelope with the cell membrane as well as nonenveloped viruses, such as poliovirus, effectively promote the coentry of toxins (8). Since the toxins most commonly used in the permeabilization assays, such as the 16.8-kDa RNase α-sarcin, severely arrest cellular protein synthesis (6, 12), they represent excellent tools for investigating the permeabilization event induced by viruses and therefore the mechanism of virus entry.

In this work, we have characterized the rotavirus-induced early permeabilization of monkey kidney (MA104) cells to toxins in an effort to learn about the initial virus-cell interactions. We found that the virus-mediated coentry of α-sarcin depends on the entry of infectious virus, is cell specific, and is not blocked by drugs that affect endocytosis or the activity of the vacuolar proton-ATPase. Also, we found that increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) did not block the coentry of α-sarcin or the onset of viral protein synthesis.

MATERIALS AND METHODS

Cells and viruses. MA104 and L929 cells (L cells) were cultured in Eagle's minimal essential medium (MEM); HEp-2 cells were grown in RPMI 1640 medium. Both culture media were supplemented with 10% fetal bovine serum. Rotavirus RRV was obtained from H. B. Greenberg, Stanford University, Stanford, Calif.; rotavirus SA114S (clone 3) was provided by M. K. Estes, Baylor College of Medicine, Houston, Tex. RRV and SA114S viruses were propagated in MA104 cells as previously described (13). Reovirus serotype 1 was obtained from C. Ramos, Instituto Nacional de Salud Pública, Cuernavaca, Morelos, Mexico, and was grown in L cells.

To prepare semipurified virus, virus-infected cells were harvested after complete cytopathic effect was attained, the cell lysate was extracted with Freon, and the virus was pelleted through a 4-ml cushion of 35% sucrose in TNC buffer (10 mM Tris-HCl [pH 7.5], 140 mM NaCl, 10 mM $CaCl_2$) by centrifugation for 2 h at 26,000 rpm and 4°C in an SW28.1 rotor (Beckman). The virus pellet was resuspended in TNC buffer and kept at 4°C until use. To obtain non-trypsin-cleaved rotavirus, the cells were washed three times with phosphate-buffered saline (PBS, pH 7.2) after adsorption of the trypsin-treated virus, and the virus preparation was kept on ice at all times during harvesting. The titer of the infectious virus preparations was obtained by an immunoperoxidase focus assay with MA104 cells as previously described (29). Titers are expressed as focus-forming units (FFU) per milliliter.

To prepare a lysate of reovirus, monolayers of L cells were infected for 1 h at 37°C with reovirus serotype 1. After this time, the inoculum was removed and MEM containing 2% fetal bovine serum was added, and the cells were kept at 37°C until complete cytopathic effect was reached. The titer of infectious virus was obtained by the immunoperoxidase focus assay with L cells and a polyclonal antibody against reovirus serotype 1 (kindly provided by T. Dermody, Vanderbilt Medical School, Nashville, Tenn.).

Permeabilization assay. Confluent monolayers of MA104 cells in 96-well tissue culture plates were infected with 10 FFU of trypsin-activated (unless otherwise indicated) rotavirus per cell in the presence of various concentrations of toxin. At 1 h after adsorption at 37°C, the virus inoculum and the toxin were removed, and fresh serum-free MEM was added for an additional period of 30 min. After this time, the medium was replaced with methionine-free MEM supplemented with 25 μCi of the ^{35}S-protein labeling mix EXPRE^{35}S^{35}S (>1,000 Ci/mmol; Dupont) per ml, and the cells were incubated for 1 h at 37°C. After the labeling period, the cells were washed with PBS, treated with 5% trichloroacetic acid (TCA) for 5 min at room temperature, and washed three times with ethanol. The cell monolayer was allowed to dry under a lamp before the addition of 50 μl of 0.1% sodium dodecyl sulfate (SDS) in 0.1 N NaOH. Total radioactivity in the sample was determined by liquid scintillation counting by solubilizing the sample in Ecolite (ICN). The toxins abrin, restrictocin A, and α-sarcin were purchased from Sigma Chemical Co.

PAGE. To analyze the ^{35}S-labeled proteins by SDS-polyacrylamide gel electrophoresis (PAGE), permeabilization assays were performed in 24-well tissue culture plates basically as described above but with some modifications. After the ^{35}S-labeling period, the cell monolayers were washed twice with PBS and dissolved in 150 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 20 μg of phenylmethylsulfonyl fluoride per ml). Nuclei and cell debris were pelleted at 5,000 rpm in an Eppendorf centrifuge for 10 min, and 10 μl-aliquots of the supernatant were mixed with an equal volume of Laemmli sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 2% β-mercaptoethanol, 10% glycerol). Samples were boiled for 3 min, and the proteins were separated by electrophoresis on SDS–11% polyacrylamide gels with the discontinuous buffer system of Laemmli. After electrophoresis, the gels were treated for fluorography with 1 M sodium salicylate and exposed to film at −70°C.

Virus treatments. (i) Preparation of double-layer particles. Aliquots of semipurified RRV virus were incubated with 50 mM EDTA for 30 min at 37°C or mock treated with PBS. Removal of the virus outer capsid was assessed by SDS-PAGE and Coomassie blue staining of the treated and nontreated viral particles, and the titer of the EDTA-treated virus was determined as described above.

(ii) Protease treatments. SA114S rotavirus obtained in the absence of trypsin was digested with 10 μg of diphenylcarbamyl chloride-treated trypsin (Sigma) per ml, 1 μg of *Asp*N (Boehringer Mannheim Biochemicals; sequencing grade) per ml, or 2 μg of α-chymotrypsin (Boehringer; sequencing grade) per ml for 30 min at 37°C. Half of the *Asp*N- and α-chymotrypsin-digested virus preparations were further treated with 10 μg of trypsin per ml for 30 min at 37°C.

(iii) MAb treatments. Immediately before the permeabilization assays, RRV rotavirus (10 FFU/cell) was preincubated for 1 h at 37°C with a 1:100 dilution of ascites of neutralizing monoclonal antibodies (MAbs) M14, M11, 1A9, 7A12, and 159 or nonneutralizing MAbs 129/60 and 255/60 or with a 1:150 dilution of neutralizing MAb 2G4. These MAbs were kindly provided by H. B. Greenberg, Stanford University. Nonneutralizing MAb HS2 was used at a 1:100 dilution and was generously supplied by Luis Padilla-Noriega, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, Mexico. The neutralization titers of these MAbs against RRV, as determined by an immunoperoxidase focus reduction assay (29), were 1:25,600 for MAbs 159, 2G4, 7A12, and 1A9; 1:3,200 for MAb M14; 1:400 for MAb M11; and <1:200 for MAbs 129/60, 255/60, and HS2.

Cell treatments. Monolayers of MA104 cells were treated prior to infection with 20 mU of *Arthrobacter ureafaciens* neuraminidase (Sigma) per ml for 1 h at 37°C as previously described (32) or with various concentrations of bafilomycin A1 (BFLA1), dansylcadaverine, cytochalasin D, A23187, ionomycin, or thapsigargin (Sigma). The drug-treated cells were used to determine the infectivity of the virus and the ability of the viral particles to permeabilize the cells to α-sarcin; in these experiments, the drugs were maintained at the appropriate concentrations during the adsorption period.

Determination of the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was measured with the fluorescence indicator fura-2. MA104 cell monolayers were trypsinized, washed twice with serum-free MEM, and resuspended in loading buffer (132.4 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, 1 mM sodium pyruvate, 1% bovine serum albumin) containing 10 μM fura-2/AM (Molecular Probes, Eugene, Oreg.). Following a 60-min incubation at 37°C, the cells were washed twice and resuspended at 2×10^6 cells/ml in loading buffer without bovine serum albumin. Fluorescence was measured at 37°C with continuous stirring in an Aminco 8000C spectrofluorimeter (SLM Instruments, Urbana, Ill.). The excitation and emission wavelengths were fixed at 340 and 510 nm, respectively. The $[Ca^{2+}]_i$ was calculated with the equation of Tsien et al. (50), modified by Guerrero and Darszon (24), with an apparent K_d for fura-2 of 224 nM (34). To calibrate the fluorescence signal of the fura-2 associated with the cells, minimum fluorescence and maximum fluorescence were determined by releasing fura-2 from the cells with 0.05% Triton X-100 in the absence of extracellular calcium and by the subsequent addition of 2 mM $CaCl_2$ to the permeabilized cells. To quench fura-2 fluorescence, 6 mM $MnCl_2$ was added.

RESULTS

Rotavirus infection mediates the entry of toxins. It has been shown for several virus families that the entry of viral particles into the cell induces an early permeabilization of the cell membrane. This permeabilization has been detected, among other methods, by monitoring the coentry of toxins with the virus during cell infection. In this work, we tested if rotaviruses were able to promote the permeabilization of MA104 cells to abrin, restrictocin A, and α-sarcin. These three toxins are potent inhibitors of the cell translation machinery, and their activity in the cytosol results in a severe arrest of protein synthesis. When added in the absence of virus, the toxins, at the concentrations tested, had a slight or no effect on the protein synthesis of MA104 cells, as measured by the incorporation of ^{35}S-labeled amino acids. However, when they were added in the presence of 10 FFU of rhesus monkey rotavirus RRV per cell, a drastic reduction in protein synthesis was observed (Fig. 1). Abrin was about 1,000-fold more active than the other two toxins, although, as was the case for restrictocin A, there was some leakage into the cells in the absence of virus. Since α-sarcin showed a more strict dependence on the presence of virus to

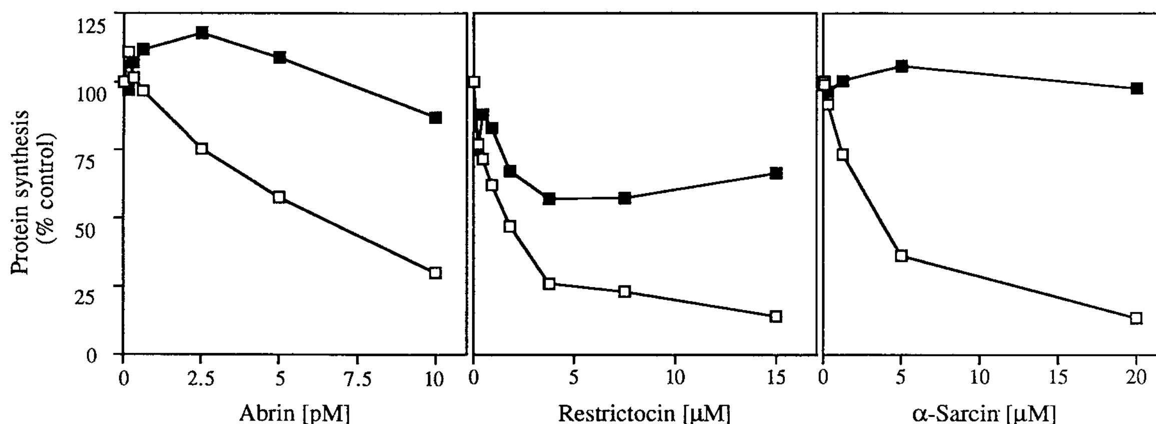

FIG. 1. Rotavirus infection mediates the entry of toxins. MA104 cells in 96-well plates were mock infected (closed squares) or infected (open squares) with 10 FFU of trypsin-activated RRV virus per cell in the presence of the indicated concentrations of abrin, restrictocin A, or α-sarcin. After 1 h of adsorption at 37°C, the virus inoculum was removed and fresh serum-free MEM was added for 30 min. The cells were then labeled with EXPRE^{35}S^{35}S for 1 h at 37°C, and the radioactivity present in the TCA-precipitable material was estimated. Data are expressed as a percentage of the ^{35}S incorporation of control cells infected or mock infected in the absence of toxins. The values represent the means for duplicate wells.

enter the cell and was able to inhibit by more than 80% cell protein synthesis when RRV was added, this toxin was used in all subsequent experiments to characterize the specificity of the coentry of toxins mediated by rotavirus.

Rotavirus-induced cell permeabilization is virus dose dependent. The effect of the virus multiplicity of infection (MOI) on the permeabilization event was determined in the range of 0.1 to 25 FFU of RRV virus per cell with 7.5 μM α-sarcin. There was already inhibition of the incorporation of ^{35}S-labeled amino acids into proteins when the cells were incubated with as little as 0.1 FFU/cell, and this inhibition became more pronounced as the MOI increased to 10 (Fig. 2). Protein synthesis was inhibited to about 50% that in control cells (mock-infected cells incubated with toxin) when RRV at an MOI of 1 was used, showing a good correlation between the coentry of toxin and the addition of infectious viral particles.

Coentry of α-sarcin is dependent on infectious virus. To determine if the entry of α-sarcin is promoted by infectious rotavirus particles and not by noninfectious physical particles, several conditions known to affect the infectivity of the virus were tested for their effect on the coentry of toxin.

(i) EDTA treatment. Treatment of infectious rotavirus particles with calcium-chelating agents is known to remove surface proteins VP4 and VP7, with the consequent abolition of virus infectivity (10, 43). In this work, treatment of semipurified RRV virus with 50 mM EDTA for 30 min at 37°C reduced virus infectivity by more than 4 log units (from 1×10^8 FFU/ml to $<0.5 \times 10^4$ FFU/ml). The EDTA-treated virus failed to promote the coentry of α-sarcin when used at a concentration equivalent to an MOI of 10 for the untreated virus (Fig. 3A). These results indicate that the entry of toxin into the cell requires the presence of VP4 and VP7 assembled into the virus surface.

(ii) Sialic acid removal from the cell surface. Most rotaviruses of animal origin initially attach to the cell surface through a sialic acid-containing cell receptor, and treatment of cells with neuraminidase greatly reduces the binding of viral particles, with the consequent reduction of virus infectivity (19, 28, 32, 51). To test if the permeabilization of cells requires the binding of virus to the cell surface, we assayed the rotavirus-mediated entry of toxin in cells that had been treated with neuraminidase in conditions that abolish the infectivity of the virus by 80 to 90% (32). Neuraminidase-treated and untreated cells were infected with RRV virus at an MOI of 10 in the presence of different concentrations of α-sarcin. In contrast to the permeabilization observed in untreated cells, cells devoid of sialic acid were not permeabilized to the toxin (Fig. 3B), indicating that the specific attachment of virus to the cell surface is necessary to modify the permeability of the membrane.

(iii) Protease treatment. The incubation of rotaviruses with trypsin activates virus infectivity through the specific cleavage of the VP4 protein in three arginine residues located at amino acid positions 231, 241, and 247 (3, 30). It has been shown that the cleavage of VP4 is required for entry of the virus into the cell, since viruses with an uncleaved protein attach to the cell surface as efficiently as cleaved virions but are not infectious (9, 20, 27). Therefore, to determine if virus penetration was the step associated with the coentry of α-sarcin, we produced RRV virus under conditions in which most VP4 molecules are not cleaved. This RRV preparation was activated in vitro with trypsin, and its infectivity was determined; the untreated virus

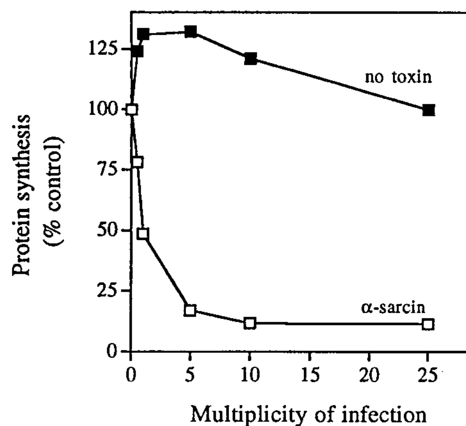

FIG. 2. Cell permeabilization induced by rotavirus is dose dependent. MA104 cells in 96-well plates were infected with the indicated MOI of trypsin-activated RRV virus in the presence or absence of 7.5 μM α-sarcin. Cells were then labeled, and the radioactivity in the TCA-precipitable material was determined as described in Materials and Methods. Data are expressed as a percentage of the ^{35}S incorporation of control cells mock infected in the absence of α-sarcin. The values represent the means for duplicate wells.

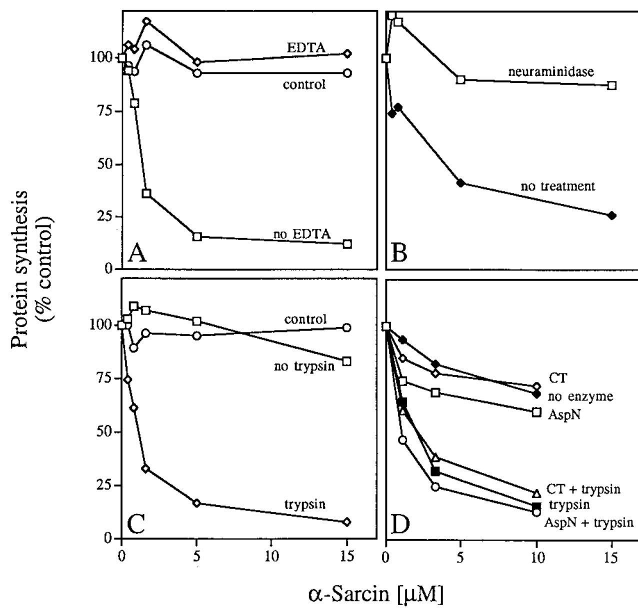

FIG. 3. Coentry of α-sarcin is mediated by infectious rotavirus. (A) MA104 cells in 96-well plates were infected with 10 FFU of trypsin-treated RRV virus per cell (no EDTA) or with an identical volume of the same RRV virus preparation treated with EDTA (EDTA) as described in Materials and Methods; mock-infected cells with MEM containing EDTA served as the control. (B) MA104 cells in 96-well plates were either not incubated or incubated with 20 mU of *A. ureafaciens* neuraminidase per ml for 1 h at 37°C. After this treatment, the cells were washed and infected with 10 FFU of trypsin-activated RRV virus per ml. (C) MA104 cells in 96-well plates were infected either with a preparation of RRV virus which was propagated in the absence of trypsin (no trypsin) or with an equivalent volume of the same virus preparation which was trypsin activated in vitro (trypsin) and contained 10 FFU/cell; control cells were mock infected with MEM containing trypsin. (D) MA104 cells in 96-well plates were infected with rotavirus SA114S which was propagated in the absence of trypsin (no enzyme) or digested with 10 μg of trypsin per ml (trypsin), 1 μg of *Asp*N per ml (AspN), or 2 μg of α-chymotrypsin per ml (CT) for 30 min at 37°C. Half of the *Asp*N- and α-chymotrypsin-treated viruses were further treated with 10 μg of trypsin per ml for 30 min at 37°C (AspN + trypsin and CT + trypsin, respectively). These protease-treated viruses were used in a permeabilization assay with 10 FFU of trypsin-treated SA114S virus per cell or the equivalent amount (by volume) of all other virus preparations. For all the conditions described above, α-sarcin was added together with the virus at the indicated concentrations. After 1 h of adsorption at 37°C, the virus and the toxin were removed and fresh serum-free MEM was added for 30 min. The cells were then labeled with EXPRE^{35}S^{35}S for 1 h, and the radioactivity present in the TCA-precipitable material was estimated. Data are expressed as a percentage of the ^{35}S incorporation of control cells infected under the same conditions as the experimental cells, but in the absence of toxin. The values represent the means for duplicate wells.

had a 50-fold-lower titer than the trypsin-treated virus (5×10^5 FFU/ml versus 2.5×10^7 FFU/ml). When equal amounts by volume of trypsin-cleaved virus (MOI, 10) and uncleaved virus were tested in a permeabilization assay, the trypsin-treated virus effectively promoted the coentry of α-sarcin, reducing the incorporation of ^{35}S-labeled amino acids into proteins to about 10% that in control cells infected in the absence of α-sarcin (Fig. 3C). The slight permeabilization induced by the untreated virus was probably due to a small fraction of viral particles with an already cleaved VP4 protein.

We recently showed that treatment of simian rotavirus SA114S with proteases different from trypsin, which cleave either at only one of the three trypsin cleavage sites (protease *Asp*N cleaves SA114S VP4 at the Arg-241/Asp-242 peptide bond) or near those sites (α-chymotrypsin cleaves at Tyr-246), does not significantly increase the infectivity of the virus, although such treatment generates VP8 and VP5 polypeptide cleavage products very similar to those obtained with trypsin (3). To test if the permeabilization of cells to α-sarcin was also dependent on the precise proteolytic cleavage of the virus spike protein, rotavirus SA114S with uncleaved VP4 was prepared and incubated in vitro with α-chymotrypsin or *Asp*N. Aliquots of the virus preparations cleaved with these proteases were further incubated with trypsin, treatment that has been shown to rescue the infectivity of the otherwise poorly infectious virus (3). Virus incubated with either α-chymotrypsin or *Asp*N behaved like non-trypsin-treated virus; both reduced protein synthesis to approximately 75% that in control cells (Fig. 3D). As occurs with infectivity, the virus samples that were further treated with trypsin permeabilized cells to α-sarcin to a level similar to that obtained with the virus samples originally activated with trypsin. These data show that for the cells to become permeable to the toxin, virus not only must attach to but also must penetrate the cell.

(iv) Effect of virus-neutralizing antibodies. To further confirm that the permeabilization of the cells to α-sarcin was due to rotavirus entry, we tested a panel of neutralizing and nonneutralizing MAbs directed to both surface proteins of rotavirus RRV. Incubation of the virus with neutralizing MAbs directed either at the VP8 (MAbs M11, M14, 1A9, and 7A12) or VP5 (MAb 2G4) domains of VP4 or at VP7 (MAb 159) blocked the permeabilization of the cells to α-sarcin (Fig. 4). On the other hand, incubation of the virus with nonneutralizing MAbs directed at VP7 (MAb 129/60), VP5 (MAb HS2), or inner capsid protein VP6 (MAb 255/60) did not prevent the inhibition of cellular protein synthesis caused by α-sarcin. The lower level at which MAb M11 blocked the coentry of α-sarcin most probably reflects its low neutralization titer (1:400) compared to those of the other neutralizing MAbs employed (1:3,200 to 1:25,600; see Materials and Methods). Since it has been shown that MAbs directed at VP8 neutralize RRV by preventing its binding to the cell, while MAbs directed at VP5 and VP7 do not affect RRV binding to the cell but rather inhibit virus entry at a postattachment step (40), these data suggest a correlation between the entry of α-sarcin and virus penetration.

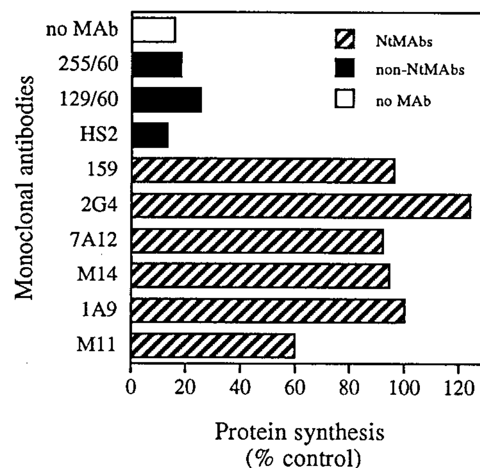

FIG. 4. Permeabilization of MA104 cells to α-sarcin is inhibited by neutralizing MAbs (NtMAbs). Trypsin-activated RRV virus (10 FFU/cell) was incubated for 1 h at 37°C with a 1:100 dilution of ascitic fluid containing the indicated MAbs, except for MAb 2G4, which was used at a 1:150 dilution. After incubation with the MAbs, the virus was adsorbed to MA104 cell monolayers in 96-well plates in the presence of 15 μM α-sarcin. The cells were labeled and processed as described in Materials and Methods. Data are expressed as a percentage of the ^{35}S incorporation of control cells infected under the same conditions but in the absence of α-sarcin. The values represent the means for duplicate wells.

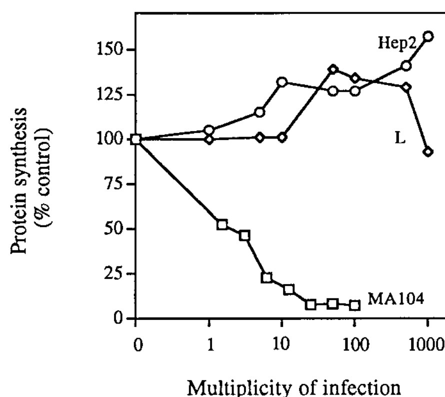

FIG. 5. Coentry of α-sarcin correlates with the cell specificity of rotavirus infection. MA104, HEp-2, and L cells in 96-well plates were infected with the indicated amounts of trypsin-activated RRV virus (infectious titer determined on MA104 cells) in the presence of 10 μM (for HEp-2 and MA104 cells) or 0.1 μM (for L cells) α-sarcin. The concentration of α-sarcin used for L cells was lower than that used for the other cell lines since L cells are more susceptible to the toxins (23) (data not shown). The cells were labeled and processed as described in Materials and Methods. Data are expressed as a percentage of the ^{35}S incorporation of control cells mock infected in the presence of α-sarcin. The values represent the means for duplicate wells.

Coentry of α-sarcin correlates with the cell specificity of rotavirus infection. Rotaviruses bind to a wide variety of cell types, although they productively infect only a subset of these cells (5). To test the specificity of the rotavirus-induced cell permeabilization, we determined if two cell lines (HEp-2 and L cells) that are poorly permissive for rotavirus infection could become permeable to the toxin when incubated with the virus. These cell lines have been shown to effectively bind rotavirus, although they are approximately 1,000 times less susceptible than MA104 cells to virus infection (data not shown; see also reference 4). The block in infectivity seems to be, at least for L cells, at the level of penetration, since liposome-mediated transfection of the virus into these cells results in efficient viral replication (5). Various amounts of RRV virus were added to either L or HEp-2 cells in the presence of α-sarcin. The addition of up to 1,000 FFU/cell (infectious titer determined on MA104 cells) did not permeabilize HEp-2 or L cells, while MA104 cells became almost totally permeabilized at an MOI of 10 (Fig. 5). These results support the specificity of the virus-cell interaction that leads to the entry of α-sarcin and the correlation between virus penetration and the entry of toxin.

Entry of α-sarcin is concomitant with virus entry. To study if the early membrane permeabilization of MA104 cells to α-sarcin is concomitant with virus entry or if the penetration of the virus leaves the cells permeable for some time, the effect on protein synthesis of adding α-sarcin at different times postinfection was determined. In this experiment, cells were infected with 10 FFU of RRV virus per cell for 30 min at 37°C, the unbound virus was removed, and the cells were either incubated or not incubated with rotavirus-neutralizing MAb 159 for 15 min at 37°C. This MAb has been shown to efficiently neutralize virus already attached to the cell surface (27). After the neutralization step, the toxin was added at different times for a period of 30 min, and the cells were labeled with ^{35}S-amino acids for 1 h and processed as described in Materials and Methods. Time zero was arbitrarily set at the end of the neutralization step. When no neutralizing MAb was added, the addition of α-sarcin completely prevented the synthesis of proteins, and the toxin was able to exert its inhibitory effect even when added 60 min after the neutralization step (Fig. 6). When α-sarcin was added at time 120 min, it no longer prevented the synthesis of proteins, and some of the viral polypeptides were readily detected. In contrast, when the virus on the surface of the cell was neutralized with MAb 159, α-sarcin did not significantly affect protein synthesis, even when added at time zero, indicating that its entry was prevented. In control experiments, in which α-sarcin was not added, the addition of a neutralizing MAb caused a delay in the synthesis of viral proteins with respect to controls without MAb treatment, in which some viral proteins could be distinguished even at time zero. These results suggest that the entry of toxin is simultaneous or very close in time to the entry of virus and also indicate that when not neutralized, infectious RRV particles continue to enter the cell for as long as 60 min after adsorption at 37°C.

Coentry of α-sarcin is not affected by inhibitors of endocytocis. Rotavirus particles have been reported to enter the cell by both direct cell membrane penetration and receptor-mediated endocytosis (20, 27, 46, 47). Although the available evidence is more compatible with the hypothesis that infectious particles enter the cell by direct penetration, some debate still exists as to whether the infectious virus cannot use the endocytic route. To determine if the internalization of α-sarcin is mediated by the virus that enters the cell through the endocytic route, we tested the effect of dansylcadaverine and cytochalasin D on the permeabilization of cells to toxin. Under the conditions assayed, neither drug affected the coentry of α-sarcin, and as previously reported, these drugs did not have a significant effect on the infectivity of rotavirus (Table 1). In contrast, reovirus infectivity was decreased by 76 and 92% when the cells were incubated with 20 μM cytochalasin D and 2 mM dansylcadaverine, respectively.

Rotavirus infection and coentry of α-sarcin do not depend on the activity of the vacuolar proton-ATPase. It has recently been reported that inhibitors of the vacuolar proton-ATPase, such as BFLA1, block infection by animal viruses that enter the cell through receptor-mediated endocytosis and require an acidic endosomal pH or a proton gradient across the membrane of the endocytic vesicle (7, 37). Since the potential role of such a proton gradient for rotavirus infection has not been evaluated, we determined the effect of BFLA1 on the infectivity of RRV virus and the coentry of α-sarcin. The addition of BFLA1 to MA104 cells at concentrations of up to 8 μM did not modify the infectivity of RRV (Table 1), while it has been reported that the drug completely blocks the infectivity of reovirus type 1 at 5 μM (21) (Table 1). Similarly, incubation of MA104 cells with 4 μM BFLA1 did not prevent the arrest of protein synthesis caused by α-sarcin in the presence of RRV (Fig. 7), suggesting that the entry of both virus and α-sarcin is independent of the activity of the vacuolar proton-ATPase.

Increases in the $[Ca^{2+}]_i$ do not affect rotavirus uncoating. We investigated if increases in the $[Ca^{2+}]_i$ alter the penetration and uncoating of the virus and/or the coentry of α-sarcin. For this, three chemicals known to raise the $[Ca^{2+}]_i$ were used, the calcium ionophores A23187 and ionomycin and the specific inhibitor of the endoplasmic reticulum (ER) Ca^{2+}-ATPase pump, thapsigargin.

In toxin permeabilization assays, MA104 cells were preincubated for 5 min with 8 μM ionomycin or 2 μM A23187 or for 15 min with 1 μM thapsigargin, and the drug concentrations were maintained during the virus adsorption period of 60 min at 37°C. Under these conditions, the drugs did not prevent the arrest in protein synthesis caused by α-sarcin in the presence of RRV virus (Fig. 8). The total incorporation of ^{35}S-amino acids in control cells treated with ionomycin or thapsigargin in the presence or absence of virus was very similar to that observed in cells not treated with the drugs. In contrast, treatment of the

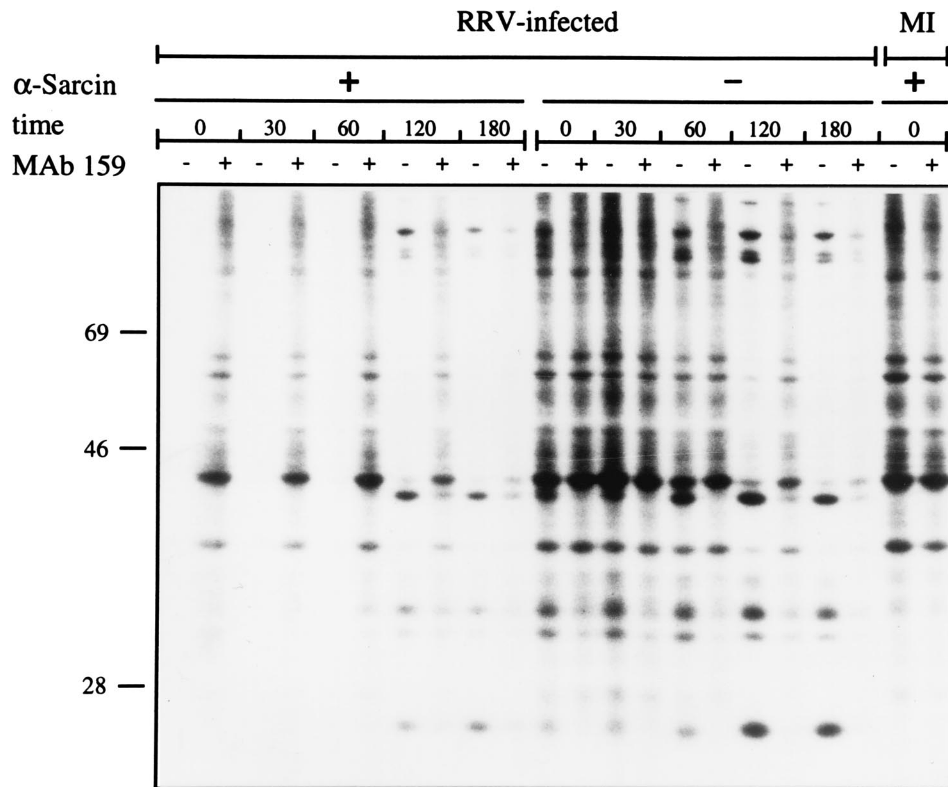

FIG. 6. Entry of α-sarcin is concomitant with virus entry. MA104 cells in 24-well plates were mock infected (MI) or infected with trypsin-activated RRV virus (10 FFU/cell) for 30 min at 37°C. After this time, the unbound virus was removed and MAb 159 (diluted 1:2,000) was added (or not) for 15 min. After removal of excess antibody (which was set as time zero), 7.5 μM α-sarcin was added (or not) at different times postneutralization for 30 min. The cells were labeled and processed as described in Materials and Methods, and total protein synthesis was analyzed by SDS-PAGE and fluorography.

cells with the ionophore A23187 resulted in a decrease in the incorporation of ^{35}S-amino acids of about 50% compared to that in untreated cells. Nevertheless, it was possible to observe the arrest in protein synthesis caused by the addition of α-sarcin and virus. The addition of 3 μM ionomycin to fura-2-loaded MA104 cells caused an ~3.8-fold increase in the $[Ca^{2+}]_i$. The mean basal $[Ca^{2+}]_i$ ± the standard deviation was found to be 280 ± 63 nM, while the $[Ca^{2+}]_i$ after the addition of ionomycin was 1,065 ± 95 nM. The $[Ca^{2+}]_i$ in cells treated with either A23187 or thapsigargin was not determined, but for the latter drug, it has been reported that its addition to MA104 cells raises the $[Ca^{2+}]_i$ to levels above the basal concentration (34). Taken together, these results indicate that the coentry of toxin is not affected by increases in the $[Ca^{2+}]_i$.

Since the coentry of α-sarcin did not seem to be affected by the increase in the $[Ca^{2+}]_i$, we studied the effect of the ionophores and thapsigargin on the synthesis of viral proteins. For this purpose, MA104 cells were preincubated for 5 min with 8 μM ionomycin or 2 μM A23187 or for 15 min with 0.5 μM thapsigargin and then infected with 10 FFU of RRV virus; the drug concentrations were maintained during the 60-min adsorption period. The unbound virus was removed, and the cells were incubated with neutralizing MAb 159 for 15 min at 37°C. This neutralization step was included to rule out the possibility that the viral protein synthesis detected was due to virus that remained attached to the cell surface and that entered the cell after the drugs were washed away from the medium. At 3 h after the neutralization step, the cells were labeled with ^{35}S-amino acids for 1 h and processed for SDS-PAGE as described in Materials and Methods. At 4 h postinfection, the synthesis of viral proteins in the presence of ionomycin, A23187, or thapsigargin could be readily detected at levels similar to those in cells infected in the absence of the drugs. In cells treated with A23187, the total synthesis of proteins was reduced; however, it was possible to detect the synthesis of viral proteins in cells infected in the presence of this ionophore (Fig. 9). We did

TABLE 1. Effect of inhibitors of endocytocis and BFLA1 on virus infectivity and α-sarcin coentry

Drug and concn	Rotavirus RRV		Reovirus type 1 % infectivity[a]
	% Infectivity[a]	% α-Sarcin coentry[b]	
BFLA1 (μM)			
2	94	20	ND[c]
4	96	18	ND
5	ND	ND	<0.01
Cytochalasin D (μM)			
1	102	8	49
10	97	6	20
20	94	ND	24
Dansylcadaverine (mM)			
0.2	108	23	48
2	100	23	8

[a] Expressed as a percentage of the FFU observed in control cells infected in the absence of drugs.
[b] ^{35}S incorporation of cells incubated with the indicated drugs in the presence of 10 μM α-sarcin, expressed as a percentage of the ^{35}S incorporation of control cells incubated with the drugs but not the toxin.
[c] ND, not determined.

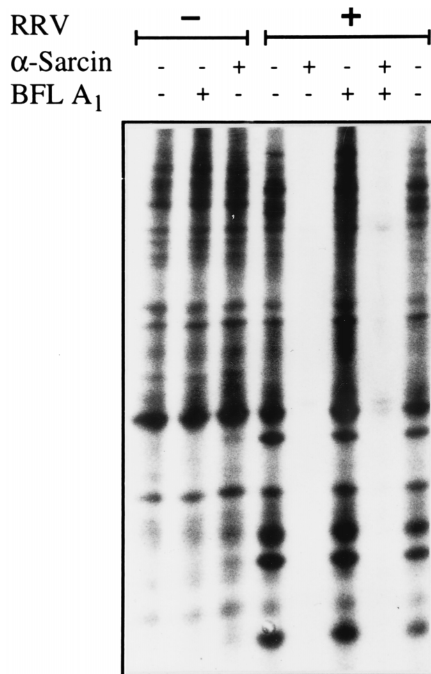

FIG. 7. Coentry of α-sarcin is not affected by BFLA1. MA104 cells in 24-well plates were preincubated for 30 min at 37°C with 4 μM BFLA1, and then 10 FFU of trypsin-activated RRV virus per cell containing or not containing 7.5 μM α-sarcin was added. After 30 min, BFLA1, α-sarcin, and virus were removed, and fresh serum-free MEM was added for 3 h. The cells were labeled for 1 h and processed as described in Materials and Methods, and total protein synthesis was analyzed by SDS-PAGE and fluorography.

not observe the altered electrophoretic migration of glycoproteins NSP4 and VP7 reported by Michelangeli et al. (34) for cells treated with thapsigargin. A possible explanation for this discrepancy might be the time of addition of the drug and the time of labeling and harvesting of the cells; they added thapsigargin 1 h postinfection and labeled the cells for 2 h beginning at 4 h postinfection.

The effect of the calcium ionophores and thapsigargin on the infectivity of the virus was also tested. The cells were infected with RRV virus in the presence of various concentrations of either ionophore or thapsigargin under the same conditions as those described above; at 12 h postinfection, the cells were fixed and immunostained for peroxidase forming units. The infectivity of RRV was not significantly affected by these compounds (data not shown). Although thapsigargin did not inhibit viral protein synthesis when present before and during the virus adsorption period, the yield of RRV after one replicative cycle carried out under these conditions decreased 3-fold; it decreased 20-fold (1.3×10^5 FFU/ml in untreated cells versus 6.8×10^3 FFU/ml in thapsigargin-treated cells) when the drug was left in the medium after the adsorption period. These findings are in agreement with previous reports (34). All of these results indicate that the increase in the $[Ca^{2+}]_i$ caused by ionomycin, A23187, and thapsigargin did not alter the uncoating of the virus, allowing the consequent genome transcription and synthesis of viral proteins.

DISCUSSION

In this work, we have shown that rotavirus infection of MA104 cells induces an early cell membrane permeabilization and promotes the coentry of toxins into the cell. This early permeabilization event has been observed for several other nonenveloped and enveloped viruses, including poliovirus, reovirus, adenovirus, influenza virus, vesicular stomatitis virus, Semliki Forest virus, Sendai virus, and vaccinia virus (1, 18, 23, 25, 37). These viruses infect the cell using different mechanisms of internalization, suggesting that the early permeabilization of the cell membrane is a common event during virus infection (7, 8). Although the biological relevance, if any, of this phenomenon is not known, its study may provide information about the mechanism of virus entry.

A general method for studying virus-induced cell permeabilization has been to monitor the coentry with the virus of protein toxins that are potent inhibitors of the cell translation machinery. In the absence of a virus, these toxins are not able to cross the cell membrane because there are no specific receptors for them; thus, the entry of toxins is taken as a measure of the permeabilization induced by a virus. Although the mechanism by which this coentry occurs is not known, Carrasco (7, 8) has proposed a model in which a proton motive force is suggested to provide the energy for the entry of the virus, which might also be used for the virus-mediated translocation of the toxin.

In this study, we have shown that the rotavirus-mediated

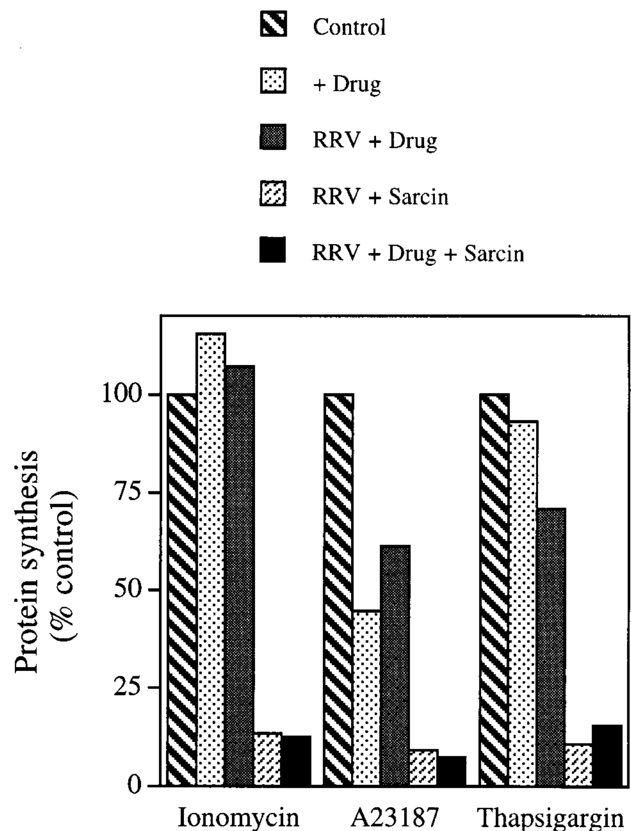

FIG. 8. Effect of the calcium ionophores ionomycin and A23187 and of thapsigargin on the coentry of α-sarcin. MA104 cells in 96-well plates were preincubated for 5 min with 8 μM ionomycin or 2 μM A23187 or for 15 min with 1 μM thapsigargin. The cells were then mock infected or infected with 10 FFU trypsin-activated RRV virus per cell in the presence or absence of 10 μM α-sarcin for 60 at 37°C; the appropriate drug concentrations were maintained during the adsorption period. The cells were labeled, and the radioactivity in the TCA-precipitable material was determined as described in Materials and Methods. Data are expressed as a percentage of the ^{35}S incorporation of control cells mock infected in the absence of α-sarcin and in the absence of drug (Control). The values represent the means for triplicate wells.

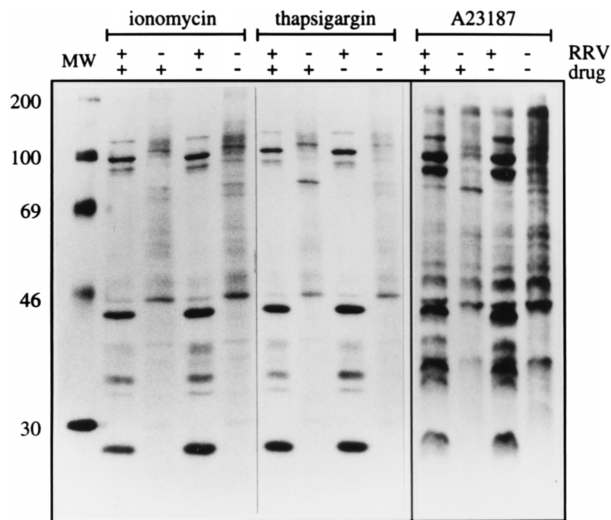

FIG. 9. Onset of viral protein synthesis is not blocked by A23187, ionomycin, or thapsigargin. MA104 cells in 24-well plates were preincubated at 37°C for 5 min with 8 μM ionomycin or 2 μM A23187 or for 15 min with 0.5 μM thapsigargin. The cells were then mock infected or infected with 10 FFU of trypsin-activated RRV virus per cell. The drugs were maintained at the same concentrations during the adsorption period of 60 min at 37°C. After this period, the unbound virus was removed and MAb 159 (diluted 1:2,000) was added for 15 min at 37°C. After the excess antibody was removed, fresh serum-free MEM was added for 3 h, and the cells were labeled for 1 h and processed as described in Materials and Methods. Total protein synthesis was analyzed by SDS-PAGE and fluorography. The samples from the A23187 treatment were run in a separate gel, which was exposed six times longer than the gel for the samples from the other treatments. MW, molecular weight.

entry of α-sarcin into MA104 cells depends on infectious virus, since various treatments that are known to prevent rotavirus infection also prevent the coentry of the toxin. Of particular interest was the observation that untrypsinized virus (which binds to but does not penetrate the cell) or virus incubated with neutralizing MAbs that inhibit a postattachment step did not promote the coentry of α-sarcin, suggesting that the permeabilization of the MA104 cell membrane is not induced by the attachment of the virus to the cell surface but by a postbinding step, presumably the penetration of infectious viral particles into the cell interior.

The permeabilization of the membrane was transient and was not detected 15 min after virus entry (the length of incubation with neutralizing MAb 159), suggesting that the potential pore or channel that might be formed during virus penetration seals quickly or, most likely, that the toxin enters the cell simultaneously with the virus, using either the same portal of entry or an independent mechanism coupled to penetration of the viral particle. The fact that neutralizing MAbs to both VP4 and VP7 inhibited the coentry of toxin suggests that both viral surface polypeptides might have a relevant role in the permeabilization event and presumably in virus entry. Whatever the mechanism of this early membrane permeabilization, the virus-cell interaction that leads to it was shown to be specific, since L cells and HEp-2 cells were not permeabilized to α-sarcin with an MOI as high as 1,000 FFU/cell, a result which correlates with the fact that these cells are at least 1,000 times less susceptible than MA104 cells to rotavirus infection.

The mechanism by which rotaviruses are internalized into cells is still a matter of debate. Biochemical and morphological approaches have been used to address this question. Ultrastructural studies have suggested that rotavirus can enter the cell by both endocytosis and direct cell membrane penetration (20, 27, 31, 39, 46, 47). However, the fact that rotavirus infection is not inhibited by lysosomotropic agents that raise the endosomal pH or by drugs that affect endocytosis or the intracellular transit of endocytic vesicles (4, 20, 27, 31) has been taken as evidence against this mode of entry or at least against the classical endocytic pathway that has been described for members of other virus families. Direct cell membrane penetration has thus been alternatively proposed as the mechanism of entry of rotaviruses. However, the evidence that supports this mechanism is rather indirect and mainly suggests that a nonendocytic route is used.

The study of the mode of entry of rotaviruses has been hampered by the lack of methods to specifically measure the entry of infectious particles. Electron microscopy and the escape of labeled virus from proteolytic digestion have been used to characterize this event (5, 31). The drawback of these methods, however, is that they cannot distinguish between the productive entry of infectious virus and the internalization of noninfectious physical particles. The inherent limitation of these methods is relevant for rotaviruses, since the ratio of infectious to noninfectious physical particles for RRV is typically about 1:100 (unpublished observations).

It has recently been reported that bovine rotavirus C486 and rotavirus RRV are able to cause fusion from without (FFWO) in MA104 cells pretreated with cholesterol (17). Also, it has been reported that bovine rotavirus RF is able to permeabilize liposomes as well as brush border-derived membrane vesicles (35, 41). These two phenomena were shown to be blocked by virus-neutralizing MAbs and to be dependent on trypsinization of the viral particle, triple-layer virus, and the amount of virus used. Thus, these events were suggested to be related to the mechanism by which rotaviruses enter cells. There are, however, some discrepancies between the conditions in which these phenomena were detected and those required for the virus to infect the cell. First, EGTA-treated, noninfectious virus was shown to mediate both FFWO and the release of carboxyfluorescein (CF) from liposomes and vesicles. Second, FFWO and neutralization were shown to be distinct events, since MAb 7A12, which neutralizes virus infectivity, did not prevent the fusion event, while nonneutralizing MAb M60, directed at VP7, did. Third, the CF release induced by rotavirus RF was inhibited by calcium at concentrations at which the virus is fully infectious. Fourth, liposomes and membrane vesicles from diverse biological origins were all permeabilized by rotavirus RF. Although the susceptibility to rotavirus infection of the cells used to obtain the vesicles was not reported, their general permeabilization, together with the fact that liposomes were also permeabilized, suggests the lack of specificity of the virus-vesicle interaction that promotes the CF release. These discrepancies were not observed in the cell membrane permeabilization to α-sarcin described in this work.

It has recently been shown that BFLA1 and concanamycin A, selective inhibitors of the endosomal proton-ATPase, strongly block the cell entry of animal viruses that require either an endosomal acidic pH or a proton gradient to infect cells (8, 26). In the case of poliovirus, cell infection is not inhibited by BFLA1, although the virus-induced coentry of α-sarcin is potently blocked by this antibiotic, indicating that an active vacuolar proton-ATPase is necessary for the permeabilization event (36). Therefore, even though previous studies have shown that rotaviruses do not require a low-pH step to infect the cell, it was of interest to study if the rotavirus-mediated delivery of toxin depended on the activity of the vacuolar proton-ATPase. Neither the infectivity of rotavirus nor the coentry of α-sarcin was found to be affected by BFLA1, suggesting that rotavirus entry and the cotranslocation of α-

sarcin are independent of both the intraendosomal acidic pH and the proton gradient across the endosomal membrane. Of interest, other viruses not affected by BFLA1 are Sendai virus, which enters the cell by fusion of its envelope with the plasma membrane (37), and the infectious subviral particles of reovirus, which have been proposed to enter the cell by direct plasma membrane penetration (23).

At some point during the process of entry of rotaviruses into the cell, the viral transcriptase is activated and the virus genome is transcribed. In vitro the transcriptase activity is observed when the surface proteins VP4 and VP7 are released from the mature triple-layer viral particles by treatment with calcium-chelating agents, converting them into double-layer particles. In vivo, it is not clear whether the viruses become uncoated during the penetration step or once they have reached the cytoplasmic milieu. Fukuhara et al. (20) have reported that during penetration of the human rotavirus strain KUN, VP4 and VP7 remain bound to the surface of the cell, in the extracellular space, while only double-layer particles gain access to the cell cytoplasm. In contrast, Ludert et al. (31) found that treatment of MA104 cells with the calcium ionophore A23187 to increase the $[Ca^{2+}]_i$ prevented the uncoating of porcine rotavirus OSU and thus suggested that the low $[Ca^{2+}]_i$ was responsible for the virus uncoating.

In this work, we tested the effect of three compounds that raise the $[Ca^{2+}]_i$ on the penetration and uncoating of rotaviruses. These drugs alter the Ca^{2+} homeostasis of the cell by different mechanisms. A23187 and ionomycin are ionophores that transport Ca^{2+} across biological membranes, including the cytoplasmic, ER, and mitochondrial membranes; in the presence of extracellular Ca^{2+}, the increase in the $[Ca^{2+}]_i$ is mainly due to an influx of Ca^{2+} from the extracellular space. Thapsigargin, on the other hand, is a specific inhibitor of the ER Ca^{2+}-ATPase pump; when this pump is inhibited, the Ca^{2+} that leaks from the ER is not resequestered and accumulates in the cytosol (48, 49). The treatment of MA104 cells with these drugs did not affect the entry of infectious RRV virus or the onset of viral protein synthesis, indicating that the low $[Ca^{2+}]_i$ is not directly responsible for the uncoating of the triple-layer particles.

In our experiments, the treatment of cells with A23187 at concentrations similar to those reported by Ludert et al. (31) resulted in a reduction in total cellular protein synthesis (about 50% of that in control, untreated cells). Nonetheless, with these conditions, we were able to detect the coentry of α-sarcin when the cells were infected with RRV. Also, we detected the synthesis of viral proteins, although at lower levels according to the reduced translation capacity of the A23187-treated cells, suggesting that the virus was able to enter the cells and to initiate the transcription-translation cycle. The discrepancy between our results and those previously reported by Ludert et al. (31) might be due to the fact that unlike us, they did not look for infectious virus, only for physical particles. It is possible that the majority of the triple-layer particles that they detected were virions that remained associated with the cell surface of the A23187-treated cells and that were equivocally scored as intracellular triple-layer virions. We looked for infectious virus, which represents a small proportion and the biologically relevant fraction of the total input virus.

Our finding that the low $[Ca^{2+}]_i$ does not seem to be the factor that triggers the uncoating of the virus is in agreement with the observations made by Ruiz et al. (42) and Gajardo et al. (22), who reported that the amount of Ca^{2+} required to solubilize the surface proteins in vitro varied depending on the rotavirus strain. For SA11 and RRV viruses, a Ca^{2+} concentration as low as 38 nM is required to convert triple-layer to double-layer particles; this concentration of Ca^{2+} is clearly lower than the basal $[Ca^{2+}]_i$ of MA104 cells (this work; 34). It is clear that the virus must become uncoated to activate the viral transcriptase, but the effector that triggers this uncoating has yet to be determined.

Recently, Dong et al. (11) reported that NSP4 added exogenously to HT29 cells mobilizes $[Ca^{2+}]_i$ through receptor-mediated phospholipase C activation and IP3 production. They hypothesized that after being released from virus-infected cells, NSP4 may bind to an unidentified molecule on the plasma membrane surface of neighboring uninfected cells to mobilize $[Ca^{2+}]_i$. Our finding that an increase in the $[Ca^{2+}]_i$ does not block the initial steps of virus infection is compatible with this hypothesis.

Although most of the available evidence is compatible with rotaviruses entering the cell by penetration at the plasma membrane level, further studies are needed to unequivocally establish the entry pathway of these viruses. In this regard, the α-sarcin coentry assay should be a valuable tool for dissecting this pathway and establishing the role of the viral proteins involved in the process.

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