Cell lines susceptible to infection are permeabilized by cleaved and solubilized outer layer proteins of rotavirus

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It has previously been shown that trypsinized triple-layered particles of rotavirus induce destabilization of liposomes and membrane vesicles in the absence of Ca2+, a condition which leads to solubilization of the outer capsid proteins of the virus. In this work, we have studied the relationship between outer capsid solubilization and permeabilization of membrane vesicles, monitoring particle and vesicle size simultaneously by changes in light scattering. Permeabilization of intact cells induced by solubilized outer capsid proteins was monitored by following the rate of entry of ethidium bromide into the cells. Solubilized outer capsid proteins separated from double-layered particles induced vesicle permeabilization. Solubilization of the outer capsid preceded and was required for vesicle or cell permeabilization. Membrane damage induced by rotavirus outer proteins was not repaired upon addition of 1 mM Ca2+ to the medium. Rotavirus infection and cell permeabilization were correlated in six different cell lines tested. This phenomenon might be related to the mechanism of virus entry into the cell. We propose a new model for rotavirus internalization based on the permeabilizing ability of outer capsid proteins and the cycling of trapped calcium in the endosomal compartment.

Introduction

Rotaviruses are members of the family Reoviridae and are the cause of severe gastroenteritis in humans and a variety of animal species (Kapikian & Chanock, 1990). These viruses are non-enveloped and icosahedral and contain 11 dsRNA segments inside a structure formed by three concentric layers of protein capsids (Estes & Cohen, 1989; Yeager et al., 1990; Prasad et al., 1988). The most abundant protein of the outer capsid, which makes up the majority of the surface, is VP7, a glycoprotein of 37 kDa. The other component, VP4, is a non-glycosylated protein of 84–88 kDa which sticks out of the surface in the form of spikes (Yeager et al., 1994; Estes et al., 1981). VP4 is responsible for haemagglutination (Kalica et al., 1983; Mackow et al., 1988) and it has been suggested that this protein is involved in virulence and the capacity of the virus to replicate in tissue culture (Offit et al., 1986). Digestion of the VP4 molecule by trypsin results in two smaller polypeptides, VP5* (65 kDa) and VP8* (28 kDa). Rotavirus infectivity in cell culture is enhanced by trypsinization and this appears to occur in vivo in the lumen of the intestine by the action of pancreatic enzymes, prior to infection of the enterocyte (Estes et al., 1981; Ludert et al., 1996).

The first event of cell infection is thought to involve the interaction of one or more of the rotavirus outer capsid proteins with a putative receptor(s) on the plasma membrane of the host cell. However, the existence of specific protein receptors for rotavirus has by no means been demonstrated. The two outer capsid proteins, VP7 and VP4, have been implicated in binding to the target cell (Fukuhara et al., 1988; Ruggeri & Greenberg, 1991; Crawford et al., 1994) and in the infectious capacity of the virus (Matsui et al., 1989).

The mechanism of rotavirus penetration into the host cell is not known. Early evidence for an endocytosis mechanism was later criticized on the basis of the apparent lack of involvement of endosomal acidification (Bass et al., 1995; Ludert et al., 1987; Fukuhara et al., 1988). Alternatively, it has been proposed that the entry of rotavirus into the cell occurs directly through the lipid phase of the plasma membrane (Suzuki et al., 1985).
Rotavirus entry has been shown to be fast and concomitant with the release of intracellular space markers, supporting the direct entry hypothesis (Kaljot et al., 1988). Changes in permeability have also been observed in membrane model systems. The studies of interactions of purified viral particles with liposomes and with membrane vesicles loaded with the self-quenching fluorophore 6-carboxyfluorescein (CF) indicate that rotaviruses induce destabilization of the lipids of the vesicle membrane at neutral pH, leading to the release of trapped fluorescent marker (Nandi et al., 1992; Ruiz et al., 1994). This effect was attributed to the proteins of the outer capsid since it took place in low Ca$^{2+}$ concentrations which induce solubilization of these proteins (Ruiz et al., 1996). However, it is not known whether the release of external capsid proteins preceded and/or was required to induce the permeabilizing effect. Furthermore, it is not yet known which of the two outer proteins is involved. In this paper, we have studied the relationship between membrane permeabilization and the mechanism of rotavirus entry into the cell by using intestinal membrane vesicles and intact cells of diverse origin with variable susceptibility to infection. The results show that permeabilization is due to the solubilized outer capsid proteins. Susceptibility of cells to rotavirus infection may be related to the capacity of the outer capsid proteins to permeabilize the membrane of a given cell line. A model for rotavirus internalization based on the permeabilizing ability of solubilized outer capsid proteins is discussed.

**Methods**

- **Cell cultures.** MA104, HeLa and L929 cells were grown in Eagle’s minimum essential medium (MEM); HEP2 and CaCo2 cells were grown in RPMI 1640 medium. The colonic cell line HT29 was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with glucose (4.5 g/l). All culture media were supplemented with 10% FCS, l-glutamine, gentamycin, amphotericin B and penicillin. Cells were cultured in a 5% CO$_2$ incubator at 37 °C.

- **Virus preparations.** Bovine rotavirus (strain RF) was multiplied in African rhesus monkey kidney cells (MA104). The monolayers were trypsinized, washed by centrifugation and resuspended at an approximate concentration of 5 x 10$^6$ cells/ml in a medium which contained 130 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 14 mM HEPES, 0.2% albumin at pH 7.4. In some cases, a different medium was used, as indicated in the figure legends. Aliquots of 300 μl of cell suspension were incubated in the presence of 50 μM EB and fluorescence was measured at 37 °C in a spectrofluorimeter. Excitation and emission wavelengths were 365 and 580 nm, respectively. Total permeabilization of the cells was determined at the end of each assay by addition of digitonin (0.13 mg/ml). In some figures, results are normalized according to the following equation: % permeabilization = $(F_t - F_0)/(F_t - F_b) \times 100$, where $F_0$ is the background fluorescence after the step change induced by the dsRNA of the rotavirus, $F_t$ is the fluorescence at time $t$ and $F_b$ is the total fluorescence after addition of digitonin. $F_p$ is a function of the total DNA content in the cuvette, which in turn is a function of cell number (Ruiz et al., 1991).

- **Susceptibility of cell lines to rotavirus infection.** Infectious yield from different cell lines was measured by titration in MA104 cells using an immunofluorescent staining technique with a monoclonal antibody against VP6 (Liprandi et al., 1991).

- **Electron microscopy of cell suspensions.** Trypsinized MA104 cells in suspension were incubated with TLP in the presence or absence of EGTA as in the permeabilization assays. At various times, the cell–virus suspension was fixed in the cuvette by addition of an equal volume of 5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed with osmium tetroxide (1%). Fixed cells were centrifuged and the pellets embedded in Epon, sectioned with diamond knives (IVIC) and viewed under the electron microscope after staining with uranyl acetate and lead citrate.

**Results**

**Solubilized outer capsid proteins of TLP lyse membrane vesicles**

As reported earlier (Ruiz et al., 1994), treatment of the TLP...
Membrane permeabilization and rotavirus entry

suspension with EGTA induced permeabilization of membrane vesicles (Fig. 1). Since Ca\(^{2+}\) chelation by EGTA induces solubilization of the outer capsid (Ruiz et al., 1996), we separated the solubilized proteins from DLP by centrifugation (14000 \(g\) for 30 min in an Eppendorf centrifuge at 4 °C) and assayed the supernatant fraction. Addition of the supernatant to the vesicle suspension provoked immediate CF release, even in the presence of 1 mM Ca\(^{2+}\). Since this fraction did not contain DLP, as revealed by agarose gel electrophoresis (not shown), the permeabilizing effect was due to the outer capsid proteins and was independent of the presence of DLP. The resuspended pellet, which contained DLP, had a negligible effect (not shown). The amplitude of CF release by the supernatant is smaller than that of the control. This is due to the fact that only two-thirds of the supernatant was added to the cuvette. These results indicate that solubilized outer capsid proteins are solely responsible for permeabilization of the membrane vesicle.

The relationship between outer capsid solubilization and vesicle destabilization was studied by a new technique in which changes in vesicle and particle size can be measured simultaneously and independently by 90 ° light scattering. In Fig. 2(A), it is shown that vesicles induced a scattering signal that was not modified by EGTA, but was decreased by total lysis with Triton X-100 and, to a lesser extent, by permeabilization with digitonin. Triton X-100 without digitonin gave the same level (not shown). The remaining scattering signal corresponds to the lysed vesicle material. The scattering signal induced by TLP at 10 °C was stable and decreased when free Ca\(^{2+}\) was removed by the addition of EGTA (Fig. 2B). At 37 °C, the increase in the scattering signal induced by the addition of TLP was transitory. The decrease in the signal corresponds to the change of particle size in the transition from TLP to DLP that occurs spontaneously in the absence of added Ca\(^{2+}\) at 37 °C. Solubilization at 10 °C requires removal of contaminant Ca\(^{2+}\) by EGTA (Ruiz et al., 1996).

When TLP were added to a membrane vesicle suspension with or without added Ca\(^{2+}\), a further scattering signal was observed, reaching the same level in both cases (Fig. 2C). In the presence of 1 mM Ca\(^{2+}\), the signal remained stable until free Ca\(^{2+}\) was removed by EGTA. The immediate decrease of the signal corresponded to solubilization of the outer capsid proteins of TLP. A slower sigmoidal second phase followed outer capsid solubilization. As shown below this second phase corresponds to the Triton X-100-sensitive fraction, that is vesicle lysis. The addition of TLP to vesicles suspended in SAM without added Ca\(^{2+}\) induced a transitory increase in the scattering signal followed by a fast decrease due to the solubilization of the TLP outer capsid. Again, the second phase of vesicle lysis followed this process. In both curves, Triton X-100 induced a further small decrease in the signal. It should be pointed out that the signal induced by the mixture of vesicles and TLP was smaller than the sum of the signals corresponding to the individual fractions in separate experiments; this is probably due to quenching of scattering. In Fig. 2(D), it is shown that addition of 1 mM Ca\(^{2+}\) just after the release of the outer capsid did not inhibit vesicle lysis. However, after Ca\(^{2+}\) addition the lysis phase appeared to be slower. This may be related to the inhibition of outer capsid solubilization by Ca\(^{2+}\) which results in a lower concentration of solubilized protein. This is confirmed by the completion of capsid release by EGTA at the end of the experiment. Solubilization could be uncoupled from vesicle lysis by low temperature (Fig. 2E). At 10 °C, solubilization of the external viral proteins required removal of contaminant Ca\(^{2+}\) in SAM by addition of EGTA. However, the sigmoidal second decrease was not observed at this temperature. Vesicle lysis induced by the outer capsid did not occur, but was brought about by addition of Triton X-100. The scattering level attained by the addition of Triton X-100 in these conditions was the same as that observed in the control curve at 37 °C. This shows that this second phase corresponds to the changes in scattering of the vesicle fraction induced by lysis.

These experiments confirm that outer capsid solubilization of TLP preceded and was required for membrane permeabilization. Furthermore, these proteins are responsible not only for making vesicles permeant to dye, as shown in a previous paper (Ruiz et al., 1994), but for their lysis.

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Fig. 1. Solubilized outer capsid proteins from TLP induce CF release from membrane vesicles. Equal volumes of TLP and MOPS–EGTA buffer (10 mM MOPS, 10 mM EGTA, 100 mM KCl, pH 7.5) were mixed and incubated for 1 min at 37 °C to induce solubilization of the outer capsid. The mixture (60 µl) was centrifuged at 14 000 \(g\) for 30 min at 4 °C in an Eppendorf microcentrifuge (model 5415C). Supernatant (40 µl) was collected after centrifugation and aliquots (14 µl) were added to fluorophore-loaded vesicles incubated in SAM supplemented with 1 mM Ca\(^{2+}\). For the TLP control experiment, particles were treated with MOPS–EGTA buffer as above and the mixture, without centrifugation, was added to CF-loaded vesicles suspended in SAM supplemented with 1 mM Ca\(^{2+}\). Final protein concentrations were 40 and 23 µg/ml for membrane and virus, respectively.
Fig. 2. Time-course of TLP outer protein solubilization and membrane vesicle lysis. For all experiments, EGTA and Triton X-100 final concentrations were the same. Light scattering units are arbitrary. (A) Change of scattering after vesicle lysis induced by detergent. Vesicles (15 µl, 30 µg/ml final protein concentration) were added to a quartz cuvette containing 1 ml SAM at 10 °C. Addition of 5 µl EGTA (5 mM final concentration) did not modify the signal. Decreases in scattering were observed with permeabilization of vesicles by the addition of 30 µl of digitonin (4 mg/ml) and vesicle lysis with 10 µl of Triton X-100 (0-1 % final concentration). (B) Change in scattering induced by solubilization of TLP outer capsid proteins. TLP (10 µl, 9 µg/ml final concentration) were added to a quartz cuvette containing 1 ml SAM at 10 or 37 °C. EGTA (5 mM) was added to induce total outer capsid solubilization. (C) Sequential solubilization of outer capsid proteins and vesicle membrane lysis measured by light scattering. Membrane vesicles (15 µl, 30 µg/ml final concentration) and TLP (10 µl, 9 µg/ml final concentration) were added to the cuvette which contained 1 ml SAM, supplemented or not with 1 mM CaCl₂ at 37 °C. Both vesicles and TLP induced an increase in scattering. Additions of EGTA and Triton X-100 were performed to attain total solubilization of TLP outer capsid proteins and vesicle lysis, respectively. (D) Addition of Ca²⁺ just after solubilization of outer capsid of TLP did not prevent vesicle lysis. Membrane vesicles (15 µl, 28 µg/ml final concentration) and TLP (10 µl, 27 µg/ml final concentration) were added to the cuvette containing 1 ml SAM at 37 °C. Addition of 1 mM CaCl₂ was performed after the first decrease in the signal corresponding to outer capsid solubilization of the TLP. Additions of EGTA and Triton X-100 were performed to attain total solubilization of TLP outer capsid proteins and total vesicle lysis, respectively. (E) Uncoupling of TLP outer capsid protein solubilization and membrane vesicle permeabilization by temperature. Membrane vesicles (15 µl, 30 µg/ml final concentration) and TLP (30 µl, 27 µg/ml final concentration) were added to the cuvette containing 1 ml SAM at 37 °C. Additions of EGTA and Triton X-100 were performed to achieve total solubilization of TLP outer capsid proteins and vesicle lysis, respectively.
Solubilized outer capsid proteins permeabilize intact MA104 cells

For rotavirus to enter the cell, it must cross the plasma membrane either directly or at the level of the endosome. In both cases, destabilization of the membrane is required for such a large particle to cross this barrier. Therefore, we took the approach of looking at membrane permeabilization in intact cells that are susceptible or not to infection. In this case, we followed the time-course of permeabilization by measuring the entry of EB, which fluoresces upon binding to nuclear DNA.

Addition of either trypsinized TLP or DLP to a suspension of cells in the presence of EB induced a step increase of EB fluorescence that we confirmed was due to staining of the viral dsRNA (Fig. 3A). As in the case of membrane vesicles, solubilization of the outer capsid by addition of EGTA to chelate Ca²⁺ induced permeabilization of the plasma membrane of MA104 cells. We confirmed by microscopy that the increase in fluorescence corresponds to the staining of the nuclei of cells made permeable to EB (not shown). Addition of DLP had no effect whatsoever on cell permeability. Cell permeabilization was dependent on particle concentration. The effect was characterized by a decrease in the lag period and an increase in both the apparent rate constant and the amplitude. All of the cells could be permeabilized at high virus concentrations (Fig. 3A, insert). As in liposomes and membrane vesicles, permeabilization was dependent on trypsinization of the particles. Untryptsinized TLP had no effect on cell permeabilization until in situ proteolysis of the rotavirus was induced by addition of trypsin (5 μg/ml), eliciting permeabilization of the cells (results not shown).

To study the relationship between outer capsid solubilization and membrane permeabilization of intact cells, we attempted to quantify the Ca²⁺ concentration at which permeabilization occurs. For this, we suspended the cells in a set of Ca²⁺-EGTA buffers to precisely define pCa and pH (Fig. 3B). When the concentration of Ca²⁺ was above 1–2 μM, the outer capsid proteins did not solubilize and permeabilization did not occur. Between 0.8 and 1.1 μM, the lag for the onset of the effect progressively increased without an apparent change in the rate or extent of permeabilization. This also indicates that in this case, outer layer solubilization occurs before the permeabilization process and is required for destabilization of the cell membrane. This suggests that membrane permeabilization of vesicles or intact cells is a similar process.

Irreversible permeabilization by solubilized outer capsid proteins of rotavirus leads to cell death

To determine if the membrane damage induced by rotavirus outer capsid proteins in the cell membrane was transient, we monitored the extent of permeabilization by adding EB at different times after rotavirus–cell interaction in Ca²⁺-free conditions (Fig. 4). When EB was added to the cell suspension 20 min after virus addition (curve b), the attained fluorescence was of the same magnitude as that observed when EB was added before the virus (curve a). The amplitude of the signal in curve (b) corresponds to the sum of the staining of viral dsRNA and dnaDNA of the permeabilized cell fraction. Addition of Ca²⁺ to the medium 20 min after virus–cell interaction did not change the virus-induced membrane permeability to EB (curve c). This indicates that the membrane was still disrupted 30 min after addition of the virus.

Membrane damage and cell death were confirmed by electron microscopy of cells fixed after interaction with virus particles in the absence of free Ca²⁺. After 8 min of incubation with TLP in a medium containing 1 mM Ca²⁺, cells appeared normal and we could observe rotavirus particles of around 75 nm in the extracellular space, near the plasma membrane, in clathrin-coated pits and vesicles, and in endocytic vesicles in the cytoplasm (Fig. 5A). No virus particles were observed in the cytoplasm outside endocytic vesicles, nor were images observed suggesting penetration through the plasma membrane. When cells were incubated with TLP in the presence of EGTA, signs of cell death were readily apparent (Fig. 5B, C). The plasma membrane appeared to be discontinuous at many points, the cytoplasmic density was much lower, mitochondria were swollen with the cristae separated and large and abundant vacuoles were observed. No virus particles were present in either the extra- or intracellular space. This can be explained by the fact that we provoked immediate solubilization of the outer capsid in these conditions precluding the entrance of intact infectious TLP. Solubilized proteins irreversibly damaged the cells. Uninfectious DLP, which do not enter or bind to cells, were probably removed from the cell suspension during the preparation of specimens for microscopy.

Relationship between susceptibility to rotavirus infection and permeabilization by outer capsid proteins in different cell types

In order to study the specificity of the permeabilizing effect, the rotavirus–cell interaction was investigated using different cell lines with various degrees of susceptibility to rotavirus infection (Fig. 6, Table 1). Outer capsid proteins of rotavirus permeabilized MA104, HT29, CaCo2 and HeLa cells. In these cell lines, rotavirus was efficiently replicated giving titres above 10⁸ p.f.u./ml. In parallel experiments, L929 cells were neither permeabilized nor infected. The cell line HeP2, which was not permeabilized, showed low rotavirus replication, being about a hundred times less susceptible than MA104. These results suggest that cell permeabilization and rotavirus replication may be related phenomena.

Discussion

The penetration of infectious virus particles into the host cell implies the crossing of a cellular membrane (Dimmock, 1982; Hoekstra & Kok, 1989). This may take place either at the
plasmalemma, in the case of direct entry, or at the endosomal membrane, derived from the plasma membrane, in the case of endocytosis. It is now well-documented that the entry of enveloped viruses involves a fusion mechanism between the viral and cellular membranes (Carrasco, 1995). However, with the exception of adenovirus (Greber et al., 1993, 1994), the mechanisms of internalization of non-enveloped viruses, such as rotavirus, are not yet clear. Many animal viruses permeabi-
VP7 is sensitive to trypsin only when it has been previously
membrane vesicles (Charpilienne et al., 1997). The fact that
VP7 is sensitive to trypsin only when it has been previously
solubilized from VLP suggests that VP7 is not responsible for the
effects reported in this paper involving proteins from TLP.
In this case, VP4 was trypsinized, but VP7 remained intact.
This points towards VP4 as the permeabilizing protein in these
experiments. Destabilization of cell membranes by outer capsid
proteins has also been shown in cell–cell fusion and syncytia
formation (Falconer et al., 1995). This effect was attributed to
the outer capsid proteins, VP4 and/or VP7. Although this
effect has been considered as a suggestion of direct entry of the
virus into the cell, the relationship between viral protein-
induced cell fusion and virus entry was not clear.

Permeabilization of the cell membrane during rotavirus–cell
interaction has been previously shown and monitored by
measurement of the leakage of pre-loaded $^{51}$Cr (Kaljot et al.,
1988). In these previous experiments, permeabilization of the
cell membrane to $^{51}$Cr was also dependent on trypsinization of
TLP. Results were compatible with the efflux of marker
concomitant with the penetration of virus into the cell to
produce infection. These findings were interpreted as evidence
for direct entry of the virus through the plasma membrane. If
$^{51}$Cr was released from the cell at the same time as the virus
entered, one should also expect co-entry of extracellular space
markers. In our experiments, we did not observe entry of EB
with intact TLP during virus–cell interaction. The radius of the
putative pore left by virus entry may be large enough to allow
passage of $^{51}$Cr, but restrict that of EB. Release of $^{51}$Cr reached
5–10% of total loading in 10 min. During this time, most of the
virus had already entered ($t_{1/2} = 3–5$ min), yet $^{51}$Cr release
increased up to 2–4 h (Kaljot et al., 1988). This suggests that
the $^{51}$Cr permeability pathways did not reseal after virus entry
in accordance with our results. An alternative interpretation is
that permeabilization to $^{51}$Cr was induced from within by outer
capsid proteins after solubilization in the cytoplasm due to the
low Ca$^{2+}$ concentration. In our experiments, a ratio of infec-
tious particles per cell of 300 f.f.u. per cell, which was similar
to that used by Kaljot et al. (1988), induced total permeabili-
zation in a short time (10 min). We do not know at this point if
$^{51}$Cr release induced by TLP and EB entry induced by
solubilized outer capsid proteins are related phenomena. It may
well be that the same protein(s) of the outer capsid is involved
in permeabilization of the plasma membrane in both cases.

There was a correlation between cell permeabilization and
the capacity of rotavirus to infect different cell lines. MA104,
HeLa, CaCo2 and HT29 cells were permeabilized by outer
capsid proteins and were susceptible to infection, whereas
L929 cells were not permeabilized and did not replicate
rotavirus. In the case of HEp2 cells, which were not
permeabilized, low level virus replication can be observed.
Detection of particle entry by titration is very sensitive since
it is possible to detect the entry of one single particle. Thus, it
may be possible that a large number of viruses are needed to
permeabilize HEp2 cells. In contrast, a high number of particles
per cell is required to detect cell permeabilization to EB. The
correlation between membrane permeabilization induced by
the outer capsid proteins and the susceptibility of the cell line

![Figure 4](image-url)
Fig. 5. Ultrastructure of cells treated with TLP particles in the presence or absence of EGTA. In (A), cells were incubated in the presence of TLP (40 µg/ml) in a medium containing 1 mM Ca\(^{2+}\) for 8 min and fixed (see Methods). Note TLP in the extracellular space, near the plasma membrane, in clathrin-coated pits and vesicles, and in endocytic vesicles in the cytoplasm (arrows). No virus particles can be observed in the cytoplasm outside endocytic vesicles. In (B) and (C), cells were fixed after incubation with TLP in a medium containing EGTA (1.7 mM) to solubilize outer capsid proteins. Note discontinuity of the plasma membrane at several points (arrows), decrease in cytoplasmic density and swollen mitochondria, with the cristae separated, and large and abundant vacuoles. No virus particles are observed either in the extra- or intracellular space. M, mitochondria; V, vacuoles.
to infection suggests that there are common mechanisms underlying permeabilization and virus entry into the cell. The composition of the cell membrane of the various cell lines may be responsible for the difference in the interaction with viral proteins and this could be one of the limiting factors in cell infection by rotavirus.

During normal infection, the virus has to be uncoated in order to activate virus replication. However, the process of solubilization of the outer proteins cannot take place, in principle, in the extracellular medium which contains Ca\(^{2+}\) concentrations which stabilize the virus. Therefore, virus uncoating could occur in the cytoplasm of the cell, where the Ca\(^{2+}\) concentration is compatible with this process, after the TLP has crossed the plasma membrane or inside the membrane. Alternatively, uncoating could take place in an isolated intracellular compartment with low Ca\(^{2+}\) concentration. Such a compartment may well be an endocytic vesicle. Permeabilization of membranes by the outer capsid proteins is not in itself evidence for either direct or endocytic entry of the virus. Both processes would require, at a given point, destabilization of the outer capsid and the membrane in order to gain access to the cytoplasm and activate virus replication. In these two hypothetical mechanisms of entry the interaction between TLP and the plasma membrane begins by binding of the virus to a membrane receptor. In the case of the direct entry pathway, this binding could induce conformational changes in one or more of the outer capsid proteins of the TLP and expose hydrophobic domains which would interact and destabilize the lipid bilayer. The DLP would enter into the cytoplasm leaving behind, perhaps in the membrane, the outer capsid proteins, thus initiating the replication process. In this alternative, it is not clear how the virus crosses the plasma membrane and how the membrane reseals after a large hole of about 80 nm is left by passage of the particle.

The alternative model, depicted in Fig. 7, involves the endocytic pathway and appears more compatible with the permeabilization of membranes and lysis of vesicles by solubilized outer proteins. In this case, binding of the rotavirus to the receptor would induce the formation of an endocytic vesicle, thus isolating the TLP within an intracellular compartment. We may envisage that the Ca\(^{2+}\) concentration falls below the critical level for stability of the outer capsid, the virus sheds its outer proteins and these lyse the vesicle membrane. In this way, the DLP passes into the cytoplasm. The volume of the endocytic vesicle compartment is small and largely occupied by the virus particle. Given that permeabilization requires a large amount of virus, the ratio of solubilized viral proteins to membrane within the endocytic vesicle would be suitable for permeabilizing the membrane.

The decrease in Ca\(^{2+}\) concentration within the endosomal vesicle required to trigger conformational changes, capsid solubilization and vesicle lysis would be equivalent to the decrease in pH in the endosome required for decapsidation and genome translocation of other enveloped and non-enveloped viruses (Carrasco, 1995). In the Ca\(^{2+}\)-dependent endocytosis model, acidification of the endosome may not be important for

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**Fig. 6.** Permeabilization of different cell types induced by solubilized outer capsid proteins of rotavirus. Purified TLP were added to the cuvette containing cells in suspension in a medium containing 50 μM EB, 100 mM KCl, 10 mM MOPS and 10 mM EGTA, pH 7.2. Cell lines used were HT29, CaCo2, L929, HEp2 and HeLa as indicated near the curves. Curves correspond to the time-course of permeabilization to EB expressed as percentage of total normalized permeabilization. The number of cells was 5 × 10⁵ cells/ml and the virus concentration was 35 μg/ml for TLP.

**Table 1.** Infectious yield of RF rotavirus-infected cells and cell permeabilization

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell origin</th>
<th>Titre (f.f.u./ml)</th>
<th>EB entry</th>
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<tbody>
<tr>
<td>MA104</td>
<td>Foetal rhesus monkey kidney</td>
<td>7.7 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>HT29</td>
<td>Human colon</td>
<td>4.0 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>CaCo2</td>
<td>Human colon</td>
<td>6.5 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervix</td>
<td>4.2 × 10⁸</td>
<td>+</td>
</tr>
<tr>
<td>L929</td>
<td>Murine fibroblast</td>
<td>1.9 × 10⁸</td>
<td>-</td>
</tr>
<tr>
<td>HEp2</td>
<td>Human larynx</td>
<td>8.6 × 10⁸</td>
<td>-</td>
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the infectious process and this can explain the lack of effect of lysosomotropic drugs on rotavirus replication (Bass et al., 1995; Kaljot et al., 1988; Ludert et al., 1987; Fukuhara et al., 1988). Equilibration of the Ca\(^{2+}\) concentration at 1 mM across all cell membranes using the Ca\(^{2+}\) ionophore A23187 did not permit solubilization of the outer capsid of TLP and infection (Ludert et al., 1987). This is consistent with the Ca\(^{2+}\)-dependent endocytosis model.

According to this hypothesis, the factors that play a role in rotavirus infection through the endocytic pathway may be: the Ca\(^{2+}\) permeability of the vesicle membrane; the Ca\(^{2+}\) concentration at which the outer capsid solubilizes; and the interaction of the proteins with the vesicle lipid bilayer which results in lysis of the membrane. These factors, together with the presence of specific receptors, may also determine the susceptibility of a given cell type to infection by a rotavirus strain.

This hypothesis represents a novel and attractive idea and takes into account different aspects of the role of Ca\(^{2+}\) in the biology and structure of rotavirus for which no explanation has been proposed. Experiments aimed at investigating this possibility are underway. This type of mechanism could also operate in the entry of other viruses into cells.

Note added in proof. This hypothesis of Ca\(^{2+}\)-dependent endocytic entry of rotavirus is consistent with recent data on the kinetics of [Ca\(^{2+}\)]\(_{\text{endosomes}}\) (Gerasimenko et al., XXXIII International Congress of Physiological Sciences, July 1997, St Petersburg, Russia, abstract P001.18), which shows that following endocytosis, [Ca\(^{2+}\)] of endosomes decreases in 5 min from a millimolar to a micromolar range.

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Fig. 7. Hypothetical model for rotavirus entry into the target cell through a Ca\(^{2+}\)-dependent endocytosis pathway (see Discussion).

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