Rotavirus infection alters Na\(^+\) and K\(^+\) homeostasis in MA-104 cells

Jesús R. del Castillo,\(^1\) Juan E. Ludert,\(^2\) Aleida Sanchez,\(^2\) Marie-Christine Ruiz,\(^1\) Fabian Michelangeli\(^1\) and Ferdinando Liprandi\(^2\)

\(^1\)Laboratorio de Fisiología Gastrointestinal and \(^2\)Laboratorio de Biología de Virus, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 21827, Caracas 1020-A, Venezuela

Infection of MA-104 cells with the OSU strain of rotavirus induced an increase in Na\(^+\) and a decrease in K\(^+\) intracellular concentrations, starting at 4 h post-infection. These changes were not related to an inhibition of the Na\(^+/\)K\(^+\) pump since ouabain-sensitive \(^{86}\)Rb uptake was augmented in rotavirus-infected cells compared to control cells, whereas the \(^{3}\)H]ouabain binding and Na\(^+\)/K\(^+\)/ATPase activity in the cell homogenate were unaffected. Furosemide-sensitive \(^{86}\)Rb uptake (Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransport) was not modified by the infection. Passive \(^{86}\)Rb efflux and \(^{22}\)Na influx were augmented in infected cells suggesting an increase in the plasma membrane permeability. The increase in intracellular Na\(^+\) concentration might be responsible for the observed stimulation of the Na\(^+/\)K\(^+\) pump. This effect was dependent upon the synthesis of viral proteins because it was abolished by addition of cycloheximide up to 4 h post-infection. Prevention of the increase in intracellular Na\(^+\) by the use of low Na\(^+\)-containing media did not modify the pattern of protein synthesis. This suggests that changes in intracellular Na\(^+\) and K\(^+\) concentrations were not related to shutoff of cellular protein synthesis. Alterations of ion contents in the rotavirus-infected enterocytes might impair intestinal absorptive capacity before the appearance of histopathological lesions.

Introduction

Infection of cultured eukaryotic cells by a number of different animal viruses has been shown to result in the alteration of intracellular cation levels (Carrasco & Lacal, 1983; Wagner, 1984). This appears to be a rather general phenomenon in lytic infections by either enveloped or naked viruses. The alteration of the intracellular concentrations of Na\(^+\) and K\(^+\) has been related to mechanisms such as a change in the plasma membrane permeability to these ions or to an inhibitory effect on Na\(^+/\)K\(^+\)/ATPase activity (Lopéz-Rivas et al., 1987; Ulug et al., 1984; Rey et al., 1988). This effect is presumed to play a role in the induction of morphological changes in infected cells, such as the development of cytomegaly in cytomegalovirus-infected cells (Nokta et al., 1988), and to contribute to the development of c.p.e. It has been proposed that the increase in intracellular sodium concentration is involved in the shutoff of host cell protein synthesis (Carrasco & Lacal, 1983). A temporal correlation between the two phenomena has been described in cells infected with different viruses (Garry et al., 1979; Lacal & Carrasco, 1982; Frugulhetti & Rebello, 1989). In other systems, changes in intracellular Na\(^+\) and K\(^+\) levels are detected after viral inhibition of cellular protein synthesis. Therefore, they do not seem to be a primary cause of the observed inhibition (Hackstadt & Mallavia, 1982; Lacal & Carrasco, 1982; Mizzen et al., 1987; Francoeur & Stanners, 1978; Nair, 1984).

Rotavirus forms a genus within the family Reoviridae, which has emerged in recent years as a more important viral aetiological agent of infantile diarrhoea in humans and many animal species. In the host, the main target cells are the enterocytes of the small intestine, which are lytically infected by this agent. Most rotavirus isolates produce a lytic infection of cultured cells; however, persistent infections have also been observed (Carpio et al., 1981). Although the time course of viral replication and its effect on the synthesis of cell macromolecules appear to vary with different rotavirus strains, shutoff of cellular protein synthesis is generally observed (Estes & Cohen, 1989).

In this study we report that infection by a highly cytolitic rotavirus strain induces a change in Na\(^+\) and K\(^+\) concentrations of infected cells. This alteration seems to be the result of changes in the plasma membrane permeability and not of a decrease in the Na\(^+/\)K\(^+\) ATPase activity. Changes in ion concentrations do not appear to be related to the observed shutoff of protein synthesis.
Methods

Cells and virus. African green monkey kidney cells (MA-104) were grown in Eagle’s MEM supplemented with 10% foetal calf serum (FCS). Virus stocks of the OSU strain of porcine rotavirus were previously treated with trypsin (10 μg/ml, 1 h at 37 °C). Time of virus addition was taken as time zero for all experiments. After 1 h adsorption at 37 °C, cells were washed twice with PBS and replenished with maintenance medium (MEM). An m.o.i. of 10 infectious units per cell was used in all experiments.

Intracellular and extracellular volume determination. Intracellular water space was determined by a modification of the method of Kletzien et al. (1975). Cells were washed with MEM and pulsed with 5 μCi/ml of 3-O-methyl-D-[3H]glucose (OMG) and 5 μCi/ml of [14C]inulin in MEM containing 5 mM-unlabelled OMG. Monolayers were incubated in these conditions for 30 min to allow equilibration of OMG across the cell and distribution of inulin in the extracellular space. Plates were then washed with ice-cold PBS containing 0-1 mM-phloretin, to inhibit equilibrated OMG efflux, air-dried and disrupted with NaOH to extract residual radioactivity. The amount of cell-associated radioactivity was compared with radioactivity in standard volumes of [3H]OMG and [14C]inulin solutions. Intracellular water was estimated as the difference between total water (determined with [3H]OMG) and extracellular water assayed by [14C]inulin.

Intracellular ion concentrations. To measure intracellular ion contents in control and infected conditions, MA-104 monolayers were washed three times with Na+- and K+-free solution, scrapped off the dish, resuspended in the same medium and centrifuged. The pellets were resuspended in distilled water (200 μl) to lyse the cells completely. Proteins were precipitated with 10% TCA, and the contents of Na+, K+ in the supernatants were determined by flame photometry. The protein content of each sample was determined by the Coomassie blue method (Gadd, 1981). Intracellular ion contents correspond to the difference between the total measured ion contents and the extracellular ion contents. The latter values were calculated from extracellular water volume (determined by inulin space) and extracellular medium concentrations. Intracellular ion concentrations were calculated by dividing the intracellular contents by the volume of intracellular water (determined by the OMG-inulin method).

Na+/K+ ATPase activity. The Na+/K+ ATPase activity was determined in homogenates of mock- and virus-infected cells as previously described (del Castillo & Robinson, 1985). Briefly, samples of the homogenates were incubated with 5 mM-MgC12, 100 mM-NaCl and 20 mM-KCl in 50 mM-Tris-HCl, in the presence or absence of 1 mM-ouabain. The reaction was started by the addition of 5 mM-ATP-Tris. The liberated phosphate was measured as described by Forbush (1983). Na+/K+ ATPase activity corresponds to the fraction of the total activity inhibited by ouabain.

Determination of 86Rb fluxes

(i) Uptake. Confluent cultures grown in 24-well plates were infected with 0-2 ml of an appropriate dilution of virus. After 1 h adsorption monolayers were washed twice with PBS and replenished with 0-5 ml of MEM supplemented with 2% FCS. Twenty minutes before the addition of the label, the maintenance medium was removed and replaced with 0-25 ml of MEM containing 25 mM-HEPES pH 7-0, with or without drugs. Uptake at given times post-infection (p.i.) was started by substituting the MEM-HEPES medium for a similar medium containing 86Rb (2 μCi/ml). At selected times, the medium was aspirated and the cultures were quickly washed four times with ice-cold PBS. After drying, cells were dissolved with 0-2 ml of 1 M-NaOH. Following neutralization with 0-05 ml of 1 M-HCl, radioactivity was determined by scintillation counting. When appropriate, 1 mM-ouabain or 3 mM-furosemide was added to the incubation medium 20 min before addition of the label. Ouabain-sensitive and furosemide-sensitive 86Rb uptake was calculated by subtracting the uptake in the presence of ouabain or furosemide from the uptake in the absence of inhibitors, respectively. Uptake in the presence of ouabain and furosemide was considered as passive uptake. In some experiments, 86Rb uptake was also measured in a medium containing 20 mM-NaCl (low Na+ medium) prepared from individual components, using N-methyl-glucamine (110 mM) as a Na+ replacement.

(ii) Efflux. Confluent monolayers of MA-104 cells grown in 24-well plates were mock-infected or infected with rotavirus and incubated at 37 °C with MEM supplemented with 2% FCS. At 6 h p.i., the maintenance medium was removed and replaced with 0-2 ml/well of MEM-HEPES medium containing 80 μCi/ml of 86Rb. After a 2 h loading period at 37 °C, the radioactive medium was aspirated and the monolayers were washed three times with PBS. Then, 200 μl of non-radioactive MEM-HEPES medium, containing ouabain (1 mM) and furosemide (3 mM), were added. At selected times, 25 μl of incubation medium was removed for radioactivity determination. Non-radioactive medium was added to restore the original volume in the well. After the last sampling, cells were washed with ice-cold PBS, air-dried and disrupted with NaOH to extract residual radioactivity. Cell-associated radioactivity at time zero was taken as 100% and results are expressed as the percentage of radioactivity remaining inside the cell.

Passive 22Na uptake. Monolayers in control or infected conditions were washed three times with low Na+ medium and incubated in MEM-HEPES medium containing 22Na (1 μCi/ml). Uptake was measured in the presence of ouabain (1 mM) and furosemide (3 mM) to inhibit sodium movement through the Na+/K+ pump and Na+/K+/2Cl- cotransport. At given times after addition of the label, the medium was removed, cultures were washed four times with ice-cold PBS and radioactivity was determined as previously described. Uptake was linear at least during the first 3 min.

[3H]Ouabain binding. MA-104 cells were incubated in MEM-HEPES medium containing [3H]ouabain (0-1 mM) for 10 min at 37 °C. At the end of this period, incubation medium was aspirated, cells were washed three times with ice-cold PBS and cell-associated radioactivity was measured. These assays yield the so-called total binding. In order to determine the non-specific binding, samples of cells were exposed for 15 min to 5 mM-ouabain before being incubated with the labelled ouabain. The difference between the total binding and the non-specific binding provides a measurement of the specific binding.

Labelling and analysis of proteins by PAGE. Confluent monolayers, infected at an m.o.i. of 10, were overlaid with methionine-free MEM. At hourly intervals after infection the cultures were pulsed for 1 h with 25 μCi/ml of [35S]methionine. At each time, cells were collected from the flask with a rubber policeman, washed in PBS and lysed for 10 min on ice with lysis buffer (0-01 M-Tris- HCl, 0-1 M-NaCl, 2 mM-PMSF and 1% NP40). After pelleting of the nuclei at 2000 g for 3 min, supernatants were collected and stored frozen for electrophoretic analysis. SDS-PAGE on 10% polyacrylamide gels and autoradiography were performed as previously described (Gorziglia et al., 1985).

Statistics. Differences between means were evaluated by the unpaired Student’s t-test. Differences were considered significant at P < 0.05.
Results

Intracellular Na\(^+\) and K\(^+\) homeostasis in rotavirus-infected cells

The intracellular Na\(^+\) and K\(^+\) concentrations of MA-104 cells, at different times after infection with the OSU strain of rotavirus and in mock-infected cells, are shown in Fig. 1. An increase in Na\(^+\) and a decrease in K\(^+\) concentrations occurred in infected cells, starting at 4 h p.i. This effect increased with time and at 8 h p.i. intracellular Na\(^+\) concentration was threefold higher than K\(^+\) concentration. At this time, no gross morphological change in the infected cells was apparent nor was viability, estimated by trypan blue or ethidium bromide exclusion, affected.

In the normal cell, the low Na\(^+\) and high K\(^+\) concentrations are maintained by two major mechanisms, in addition to the membrane's intrinsic permeability to these ions. The first is the exchange of Na\(^+\) for K\(^+\), mediated by the Na\(^+\)/K\(^+\) pump, which is specifically inhibited by ouabain. The second is the Na\(^+\), K\(^+\), 2Cl\(^-\) cotransport system, which is sensitive to the action of diuretics like furosemide. To evaluate the relative contributions of these mechanisms to the changes in Na\(^+\) and K\(^+\) contents in cells infected by rotavirus, we studied the effect of ouabain and/or furosemide on the transport of \(^{86}\)Rb, a substitute for K\(^+\).

Fig. 2 shows the time course of \(^{86}\)Rb uptake in control and infected MA-104 cells, at 4 h p.i. In both groups, \(^{86}\)Rb uptake was linear at least during the first 2 min. Rotavirus infection produced an increase in initial \(^{86}\)Rb uptake.

In control cells, ouabain and furosemide inhibited initial \(^{86}\)Rb uptake by 46% and 33%, respectively. The uptake obtained in the presence of both drugs (considered as the passive, non-mediated uptake) represents about 19% of the total uptake. Ouabain-sensitive, furosemide-sensitive and passive \(^{86}\)Rb uptake at 4 h p.i. and 8 h p.i. are shown in Fig. 3. In infected cells the ouabain-sensitive and the passive uptakes but not the furosemide-sensitive uptake were increased. The effect was more marked at 8 h p.i. These results indicate that the changes in ion concentrations were not related to inhibition of the Na\(^+\)/K\(^+\) pump, the activity of which appeared to be stimulated, but rather to an increase in the plasma membrane permeability, as suggested by the increase in the passive \(^{86}\)Rb uptake.

To distinguish whether the increase in the activity of the pump was due to an increase in the number of sites or to stimulation of the units already present, we estimated the activity of the Na\(^+\)/K\(^+\) ATPase, the biochemical expression of the Na\(^+\)/K\(^+\) pump, and estimated the number of pumps by \(^{[3H]}\)ouabain binding. At 8 h p.i. neither the Na\(^+\)/K\(^+\) ATPase nor ouabain binding were affected by virus infection, indicating that the increased ouabain-sensitive \(^{86}\)Rb uptake was due to a stimulation of pre-existing pumps (Table 1).

The plasma membrane permeability to Na\(^+\) and K\(^+\) was evaluated by measuring the passive \(^{22}\)Na uptake and \(^{86}\)Rb efflux, respectively. As shown in Table 2 and Fig. 4 the permeability to both ions was increased in rotavirus-infected cells.

These results suggest an increase in the activity of the pump as a compensatory response to the increased Na\(^+\) concentration. To verify this hypothesis we tested the \(^{86}\)Rb influx in cells maintained, after infection, in medium with a lower Na\(^+\) concentration (20 mM). As
Fig. 3. Ouabain-sensitive (A), furosemide-sensitive (B) and passive (C) influx in mock-infected (empty bars) and rotavirus-infected (hatched bars) MA-104 cells at 4 (a) and 8 (b) h p.i. Results are mean values ± S.E.M. of three experiments. Levels of significance of the difference between control and infected cells, indicated in the figure, were evaluated by Student's t-test. NS, Not significant; *, P<0.05; **, P<0.01.

Table 1. Na⁺/K⁺ ATPase activity and specific [³H]ouabain binding in mock-infected (control) and rotavirus-infected MA-104 cells at 8 h p.i.

<table>
<thead>
<tr>
<th>Condition</th>
<th>ATPase activity* (nmol/mg protein × min)</th>
<th>[³H]ouabain binding* (pmol/mg protein × min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67.3 ± 5.38</td>
<td>1.4 ± 0.05</td>
</tr>
<tr>
<td>Infected</td>
<td>64.6 ± 4.52</td>
<td>1.5 ± 0.10</td>
</tr>
</tbody>
</table>

* Results are mean values ± S.E.M. of three determinations.

shown in Fig. 5, reduction of extracellular Na⁺ concentration from 130 mM to 20 mM decreased the total entry of ⁸⁶Rb in both control and infected cells and abolished the increase in influx caused by rotavirus infection.

Table 2. ²²Na influx in MA-104 cells infected by rotavirus

<table>
<thead>
<tr>
<th>Condition</th>
<th>⁴ h p.i.</th>
<th>⁸ h p.i.</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>6636 ± 208</td>
<td>6595 ± 283</td>
</tr>
<tr>
<td>Infected</td>
<td>8636 ± 150†</td>
<td>11225 ± 451†</td>
</tr>
</tbody>
</table>

* Results are mean values ± S.E.M. of three determinations.
† P<0.001, compared to mock-infected condition (control).

Fig. 4. ⁸⁶Rb efflux at 8 h p.i. in rotavirus-infected (●) and mock-infected (○) cells. Cell-associated radioactivity at different times is expressed as the percentage of cell-associated radioactivity at time zero. Points are the mean values of four replicate wells.

Fig. 5. Ouabain-sensitive ⁸⁶Rb uptake in rotavirus-infected cells (hatched bars) and control cells (empty bars), maintained in normal medium (130 mM-Na⁺) and low Na⁺ medium (20 mM-Na⁺). Measurements were made at 4 h.p.i. Results are expressed as mean values ± S.E.M. of three experiments. NS, Not significant; *, P<0.01 compared to control.

Fig. 2. Normal medium Low Na⁺ medium
Synthesis of proteins in rotavirus-infected cells

Modification of the intracellular cation concentrations by virus infection has been proposed as a possible mechanism associated with inhibition of cellular protein synthesis in a number of virus-cell systems (Carrasco & Lacal, 1983). We therefore studied the kinetics of protein synthesis under the same conditions (virus strain, m.o.i.) in which the effect on membrane permeability had been demonstrated. Protein synthesis in rotavirus-infected MA-104 cells, pulsed at hourly intervals with $[^{35}\text{S}]$methionine, is shown in Fig. 6. It can be seen that synthesis of viral proteins is initially detected at the second hour p.i., reaches a maximal rate at the fourth hour p.i. and declines at the eighth hour p.i. Increasing inhibition of cellular protein synthesis occurs starting at the fourth hour p.i. At this time the intracellular Na$^+$ concentration is increased by approximately 20%. To study the possible relationship between the two phenomena, protein synthesis was studied in cells maintained in low (20 mM) Na$^+$ medium. Under this condition the increase in $^{86}\text{Rb}$ influx induced by virus infection is abolished (Fig. 5). As shown in Fig. 7 the shutoff of cellular protein synthesis in infected cells, measured at 5 and 6 h p.i., was not affected by the low extracellular Na$^+$ concentration.

To evaluate whether changes in plasma membrane permeability were mediated by the synthesis of new proteins induced by rotavirus infection, the effect of cycloheximide, an inhibitor of protein synthesis, on ouabain-sensitive $^{86}\text{Rb}$ influx was determined (Fig. 8). Addition of cycloheximide up to 4 h p.i. prevented the increase in $^{86}\text{Rb}$ in infected cells. The drug did not affect the influx in uninfected cells.

Discussion

Rotavirus infection produces in both the nucleus and cytoplasm of the host cell a variety of cytopathological changes, which include a generalized inhibition of the cell macromolecular synthesis (Carpio et al., 1981). In
this report, we show that rotavirus infection of MA-104 cells leads to a modification of the intracellular monovalent cation concentrations. This change might have marked effects on the metabolism of the infected cell and may impair some of its functions before lysis or detachment from its substrate occurs. Alteration of monovalent cation levels is a common result of infection by viruses with different replication strategies including cytoplasmic naked viruses (picornavirus), enveloped viruses (rhabdovirus, alphavirus) and nuclear enveloped viruses (cytomegalovirus) (Nair, 1984; Lacal & Carrasco, 1982; Francoeur & Stanners, 1978; Garry et al., 1979; Nokta et al., 1988). Mechanisms by which homeostasis of ions is affected appear to vary between different viruses. In the case of rotavirus, we found that the mechanism that most likely accounts for the change in intracellular ion concentrations was an increase in the passive permeability to monovalent cations rather than to an inhibitory effect on the Na\(^+\)/K\(^+\) pump activity. This conclusion is supported by the observation in infected cells of (i) an increase in the passive \(^{86}\)Rb efflux and \(^{22}\)Na uptake; (ii) a stimulation of the ouabain-sensitive \(^{86}\)Rb uptake, indicating a stimulation of the Na\(^+\)/K\(^+\) pump; and (iii) no changes in the number of Na\(^+\)/K\(^+\) pumps and in the activity of the Na\(^+\)/K\(^+\) ATPase. This situation is similar to the one observed in cells infected with picornavirus or cytomegalovirus (Nair, 1984; Lacal & Carrasco, 1982; Nokta et al., 1988) but completely different from the one observed after infection with togavirus or arenavirus (Ulug et al., 1984; Rey et al., 1988), in which ion concentration changes appeared to be related to inhibition of the Na\(^+\)/K\(^+\) ATPase activity. In addition, we have found that rotavirus infection increases intracellular free Ca\(^2+\) concentration with approximately the same time course as the changes observed in monovalent cation homeostasis (Michelangeli et al., 1991). The relationship between the increase in Na\(^+\), K\(^+\) and Ca\(^2+\) permeability remains to be established.

It has been proposed that changes in the intracellular levels of Na\(^+\) and K\(^+\) are causally related to selective shutoff of cellular protein synthesis (Carrasco & Lacal, 1983). Our results with rotavirus-infected cells show that a marked shutoff of cellular protein synthesis coincided with a significant change in Na\(^+\) and K\(^+\) levels. However, prevention of the increase in intracellular Na\(^+\) concentration by the use of low Na\(^+\)-containing media did not modify the pattern of protein synthesis. In this condition the intracellular K\(^+\) concentration should remain low due to the decrease in pump activity and the supposed increase in permeability to ions. This suggests that inhibition of cellular protein synthesis by rotavirus is independent of altered monovalent cation concentrations.

The molecular basis of the virus-induced changes in membrane permeability, which implies the identification of the virus-encoded product responsible for the effect, remains to be defined. Insertion of viral proteins
in the plasma membrane has been proposed to play a role in the alteration of ion concentrations induced by coronavirus or alphaviruses (Mizzen et al., 1987; Ulug et al., 1984). One rotavirus structural protein, VP4, the product of gene 4, appears to be able in its cleaved form to interact with the cell plasma membrane affecting its continuity and allowing virus penetration (Kaljot et al., 1988). Two rotavirus proteins (VP7 and NS28, products of genes 8 or 9 and 10, respectively) are inserted into the endoplasmic reticulum membrane (reviewed by Estes & Cohen, 1989), but no evidence has been reported so far for the interaction of any rotavirus product with the plasma membrane. Alternatively, a virus product might affect membrane permeability through an indirect pathway.

Defective Na⁺ transport and/or carbohydrate malabsorption, due to extensive atrophy of the villi, have been considered as primary mechanisms in the establishment of diarrhoea induced by rotavirus infection. However, in some cases the onset of diarrhoea has been shown to precede villous atrophy (Crouch & Woode, 1978; Theil et al., 1978), suggesting functional alterations in the small intestine before the appearance of histopathological lesions. A mechanism responsible for these functional changes could be an alteration of the homeostasis of Na⁺ and K⁺ in the infected enterocytes, as is found to occur in cultured cells. The increase in plasma membrane permeability would increase the intracellular Na⁺ concentration, reducing the Na⁺ gradient across the apical membrane of absorptive cells and in this way decreasing Na⁺ and sugar absorption. Thus, rotavirus-induced impairment of intestinal absorptive capacity, preceding massive enterocyte death, could constitute an initial stage of the development of diarrhoea.

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References


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