Modification of Membrane Permeability in Poliovirus-infected HeLa Cells: Effect of Guanidine

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SUMMARY

A drastic modification in permeability to monovalent ions occurred in HeLa cells infected with poliovirus, starting in the period from the third to the fourth h post-infection. The bulk of poliovirus protein synthesis took place from the third to the sixth h in cells in which the concentration of monovalent ions in the cytoplasm had changed considerably compared to uninfected cells. Under our conditions of infection (HeLa cells grown in monolayer), poliovirus translation continued beyond the eighth h. Modification of permeability to $^{86}$Rb$^+$ ions induced by poliovirus infection was prevented by the addition of 2 mM-guanidine at the time of infection. However, when poliovirus replication was allowed to take place for 1 h before addition of guanidine, the membrane did become modified to some extent. The degree of leakiness to $^{86}$Rb$^+$ ions increased when guanidine was added later. The blockage of membrane leakiness by early addition of guanidine was overcome by the addition of choline. The inhibition of cellular protein synthesis, which occurred early in infection or in the presence of guanidine, did not coincide with the modification of permeability to $^{86}$Rb$^+$ ions. Viral protein synthesis was necessary in order to modify the membrane late in infection, since addition of $10^{-5}$ M-cycloheximide during the first 2 h of infection prevented the leakiness to $^{86}$Rb$^+$ ions observed from the fourth h after infection. Membrane potential, as measured by the lipophilic cation TPP$^+$ (tetraphenylphosphonium), dropped from the fourth h post-infection. This change in the membrane was also prevented when viral gene expression was inhibited by the presence of guanidine.

INTRODUCTION

Picornaviruses induce a drastic inhibition of host protein synthesis after infection of mammalian cells (Ackermann et al., 1959; Salzman et al., 1959; Saborio et al., 1974; Nuss & Koch, 1976; Carrasco & Smith, 1980). Two different mechanisms are probably involved, depending on the kind of virus considered (Jen et al., 1980; Lacal & Carrasco, 1982). Mengovirus and encephalomyocarditis (EMC) virus modify the permeability of the cellular membrane at the time when the decline in host protein synthesis takes place (Farham & Epstein, 1963; Carrasco & Smith, 1976; Egberts et al., 1977). No modification in the protein synthesizing machinery is observed after mengovirus or EMC virus infection, since the cell-free systems obtained from virus-infected cells were equally able to translate host and viral mRNA (Abreu & Lucas-Lenard, 1976; Jen et al., 1980). In contrast, cell-free systems obtained from poliovirus-infected HeLa cells late in infection do not translate capped mRNAs (Rose et al., 1978). This inhibition is overcome by the addition of a cap-binding factor involved in the initiation of protein synthesis (Helentjaris & Ehrenfeld, 1978; Trachsel et al., 1980).

Analysis of the cellular membrane integrity in poliovirus-infected cells indicated that a rapid increase in sodium uptake takes place 2 h after infection. It was later found that the concentration of potassium also drops between 2 and 3 h post-infection (Nair et al., 1979; Nair, 1981). This modification is prevented when guanidine is present from the beginning of the infection. We have now analysed, in parallel experiments, the rate of protein synthesis, the
86Rb+ content, the dependence of membrane modification on viral gene expression in poliovirus-infected cells and the membrane potential throughout infection. The results are presented in this contribution.

METHODS

Growth of HeLa cells and poliovirus. HeLa cells were grown in Dulbecco's modified Eagle's medium (E4D), supplemented with 10% newborn calf serum (Difco) (E4D10 medium) and incubated at 37 °C in a 5% CO2 atmosphere.

Poliovirus type I (Mahoney strain) was grown on HeLa cells in E4D2 medium. Cells were sedimented at 4000 rev/min for 20 min. The pellet was resuspended in distilled water, freeze-thawed three times and centrifuged at 4000 rev/min for 15 min. Then the supernates of both centrifugations were mixed, and the concentration of poliovirus estimated by plaque assay.

Protein synthesis. HeLa cells grown on 16 mm diam. 24-well plates (Falcon Plastics) were infected with poliovirus type I. After infection at the indicated multiplicity of infection (m.o.i.), cells were incubated with 0.2 ml E4D2 medium and at the indicated times the medium was replaced by 0.2 ml methionine-free medium supplemented with 1% calf serum (E4D1) and 0.5 μCi/ml L-[3SS]methionine (1.160 Ci/mmol, Amersham International). After incubation for the indicated period of time, the medium was removed, the cell monolayers were washed with phosphate-buffered saline (PBS) and 5% trichloroacetic acid (TCA) was added. The monolayer was then washed twice with ethanol and dissolved in 0.2 ml 0.1 N-NaOH, 1% SDS; samples of 0.15 ml were taken to measure radioactivity in an Intertechnique scintillation spectrometer.

Analysis of proteins by polyacrylamide gel electrophoresis (PAGE). The labelled proteins were analysed by PAGE as follows: after the labelling period with 10 μCi/ml L-[35S]methionine, the cells were washed with PBS solution and dissolved in 100 μl 0.02 M-NaOH, 1% SDS and 200 μl sample buffer (62.5 mM-Tris pH 6.8, 2% SDS, 0.1 M-dithiothreitol, 17% glycerol and 0.024% bromophenol blue as indicator). Each sample was sonicated to reduce viscosity and heated to 90 °C for 5 min. Ten μl were applied to 15% polyacrylamide gels and run overnight at 25 to 30 V. After treatment for fluorography, the gels were dried and exposed at -70 °C to X-ray film (Kodak). Ten μl amounts of each sample were also precipitated by 1 ml 10% TCA, heated for 5 min at 90 °C and filtered through GF/C glass fibre filters to count the total protein synthesized. Dried filters were counted in a scintillation spectrometer.

Intracellular 86Rb+ content. Confluent monolayers of HeLa cells grown on Linbro plates were incubated overnight at 37 °C in a 5% CO2 atmosphere with 0.2 μCi 86Rb+ (1 mCi/ml) per well. The cells were then infected or mock-infected with poliovirus. The intracellular 86Rb+ content was measured after washing the monolayers twice with PBS and extraction with 0.4 ml 5% TCA; 0.2 ml samples were mixed with 2 ml distilled water and Cerenkov radiation was estimated in an Intertechnique scintillation spectrometer.

Membrane potential. Cells were grown, as described, in E4D10 medium until confluent. Then the medium was replaced with 0.2 ml Eagle's medium (low potassium medium) or 0.2 ml Eagle's medium with no NaCl, but with 110 mM-KCl (high potassium medium) supplemented with 1% calf serum and 10 μM-[3H]TPP+ (tetraphenylphosphonium; 10 mCi/mmol) kindly provided by Dr R. Kaback (Roche Institute). Cell cultures were incubated for 45 min with [3H]TPP+ at 37 °C to reach the steady state level of TPP+ accumulation. At this point, poliovirus was added and cells were incubated for 1 h more.

Each hour thereafter the medium was removed and replaced by 0.2 ml 0.1 M-NaOH, 1% SDS, samples of 0.15 ml were withdrawn and radioactivity was estimated. The [3H]TPP+ present in the high potassium medium was subtracted from the radioactivity in the cells kept in low potassium medium. Appropriate corrections were performed for cellular volume variations after infection.

RESULTS

When human cells are infected with poliovirus, cellular protein synthesis is severely inhibited (Baltimore, 1969). Two h after poliovirus infection, the rate of protein synthesis in HeLa cells was reduced by 70 to 80% as compared with the uninfected controls (Fig. 1). Analysis by PAGE of the proteins synthesized in cells indicated that poliovirus proteins appeared at 2 h post-infection, were maximal from 3 to 6 h and still apparent 8 h after infection (Fig. 1). It must be emphasized that in our experiments we used HeLa cells grown in monolayer cultures and infection under those conditions lasts longer than in suspension cultures. The addition of 2 mM-guanidine at 2 h post-infection greatly inhibits the appearance of poliovirus proteins (Crowther & Melnick, 1961; Loddo et al., 1962; Rightsel et al., 1961), whereas if guanidine was added later, at 3 or 5 h post-infection, almost no inhibition of poliovirus translation occurred (Fig. 1).

Analysis of the 86Rb+ content in parallel cultures showed no variation during the first 3 h after
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Fig. 1. Effect of guanidine on protein synthesis and intracellular $^{86}$Rb$^+$ content of poliovirus-infected HeLa cells. HeLa cells incubated overnight with 0.2 μCi $^{86}$Rb$^+$ in 1 ml E$_4$D$_{10}$ were infected or mock-infected with poliovirus type I (m.o.i. ≈ 10). Each hour after the virus adsorption period, parallel cultures were pulse-labelled for 1 h with 10 μCi/ml $[^3]$S-methionine or processed for intracellular $^{86}$Rb$^+$ content as described in Methods. Analysis of the proteins synthesized was carried out by PAGE and the autoradiographs are shown at the bottom. (a) Protein synthesis in poliovirus-infected HeLa cells with no guanidine (O) or 2 mM-guanidine added at 2 h (O), 3 h (A) or 5 h (△) post-infection. (b) Intracellular $^{86}$Rb$^+$ content in infected cells with no guanidine (O) or 2 mM-guanidine added at 0 h (O), 1 h (△), 1.5 h (△), 2 h (□) or 2.5 h (□) post-infection. (c) Intracellular $^{86}$Rb$^+$ content in infected cells with no guanidine (O) or 2 mM-guanidine added at 3 h (△), 3.5 h (△), 4 h (□) and 5 h (□) post-infection. Ac, Actin; C, control.

the addition of poliovirus, and by 5 h about 50% of the $^{86}$Rb$^+$ content of the cell had been lost, suggesting that a drastic modification in the monovalent ion content of the poliovirus-infected cells had occurred. The presence of 2 mM-guanidine from the beginning of infection prevented modification of membrane permeability, suggesting that poliovirus replication and viral gene expression are in some way involved in the induction of membrane leakiness. However, even small amounts of viral products induced modifications in the permeability to $^{86}$Rb$^+$, since when guanidine was added 2 h post-infection some alteration of the membrane took place, even though the synthesis of viral proteins was greatly reduced (Fig. 1).

The inhibition of virus growth caused by guanidine is reversed by the addition of choline to the culture medium (Mosser et al., 1971; Nair et al., 1979). Fig. 2 illustrates that the presence of...
Fig. 2. Choline reversal of guanidine-induced inhibition of viral protein synthesis and intracellular $^{86}Rb^+$ release from poliovirus-infected HeLa cells. HeLa cells incubated overnight with 0.2 $\mu$Ci $^{86}Rb^+$ in 1 ml E$_8$D$_{10}$ were infected or mock-infected with poliovirus type I (m.o.i. $\approx$ 10). Poliovirus replication was inhibited by 0.4 mM-guanidine at 0 h post-infection and the inhibition was reversed at different times after infection with 20 mM-choline. PAGE of synthesized proteins was performed and autoradiographs of gels are shown. (a, b, c) Intracellular $^{86}Rb^+$ content of control poliovirus-infected HeLa cells ($\bullet$), plus 0.4 mM-guanidine added at 0 h post-infection (○) and treated with 20 mM-choline at 0 h (▲), 1 h (△), 3 h (■) or 5 h (□) post-infection. (d, e, f) Proteins synthesis in poliovirus-infected HeLa cells with symbols as for (a to c). Ac, Actin; C, control.

0.4 mM-guanidine from the beginning of infection did not prevent inhibition of host protein synthesis, although the alteration of membrane permeability did not occur. When choline was added at different times to guanidine-inhibited cells, it released the inhibition of poliovirus translation. Also, leakiness to $^{86}Rb^+$ ions developed gradually.

These experiments suggested that although viral replication is somehow involved in the appearance of membrane leakiness, small amounts of viral products are sufficient to induce alterations in the permeability of the membrane. In order to analyse this problem further we used the translation inhibitor, cycloheximide. This was added to poliovirus-infected cells at different times after infection, and the $^{86}Rb^+$ content estimated. The results in Fig. 3 show that when cycloheximide was added 2-5 h after infection, modification of the membrane took place,
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Fig. 3. Cycloheximide inhibition of $^{86}$Rb$^+$ release from poliovirus-infected HeLa cells. HeLa cells incubated overnight with 0.2 $\mu$Ci $^{86}$Rb$^+$/well in 1 ml E$_4$D$_{10}$ in 16 mm diam. 24-well plates were infected with poliovirus type I (m.o.i. $\approx$ 10). Cycloheximide was added at the indicated times after infection at a final concentration of $10^{-5}$ M. Intracellular $^{86}$Rb$^+$ content was then analysed as described in Fig. 2 every h post-infection in control poliovirus-infected HeLa cells (○) and in cells treated with cycloheximide at 0 h (○), 0.5 h (△), 1 h (△), 1.5 h (■), 2 h (□), 2.5 h (+), 3 h (●), 3.5 h (▼), 4 h (♦) and 5.5 h (◇) post-infection.

Fig. 4. Membrane potential in poliovirus-infected HeLa cells. Confluent HeLa cells were labelled with $[^{3}H]$TPP$^+$ for 45 min as described in Methods and then mock-infected or infected with poliovirus type I (m.o.i. $\approx$ 10) untreated or treated with 2 mm-guanidine. After 1 h adsorption, the cells were processed for intracellular $[^{3}H]$TPP$^+$ content and background levels obtained in high potassium medium subtracted (18 300 ct/min). (a) Control cells and (b) poliovirus-infected cells untreated (○) or guanidine-treated (●).

whereas when added 1 or 2 h after infection membrane leakiness was prevented. These results suggest that the viral products synthesized during the first 2 h of infection are not sufficient to alter the membrane and, together with the guanidine experiments, indicate that a viral protein is, perhaps, the cause of this alteration.

Lichtstein et al. (1979) have described a method to measure the membrane potential by means of the lipophilic cation TPP$^+$. We applied this method, and estimated membrane potential in human HeLa cells at various times after poliovirus infection. Fig. 4 shows that membrane potential dropped at around 4 h after infection. This drop was prevented if guanidine was present, indicating that active viral replication is necessary to induce this change in the cellular membrane. Since permeability to $^{86}$Rb$^+$ ions is also altered a possible explanation for this alteration in membrane potential is that diffusion of ions is enhanced at this time of infection. We have also observed a reduction of membrane potential early in infection. Indeed, using microelectrodes a temporary drop in membrane potential was observed after infection with Sendai virus (Levanon et al., 1977; Kohn, 1979; Pasternak & Micklem, 1981). It is possible that if this change occurs with poliovirus, it will not be observed by the TPP$^+$ technique. On the other hand, we have also observed that guanidine by itself has an effect on membrane potential, and at a concentration of 1 mM a 25% reduction in control uninfected cultures was observed (Fig. 4).
DISCUSSION

Previous studies on the modification of membrane permeability after poliovirus infection suggested that an increase in intracellular sodium ions takes place from the second h post-infection in poliovirus-infected cells (Nair et al., 1979). Later it was reported that K+ leaks from poliovirus-infected cells from 2 to 3 h post-infection (Nair, 1981). Our present results indicate that poliovirus-infected cells loaded with 86Rb+ ions start to lose them around the fourth h post-infection. This suggests that from that time membrane permeability is altered, allowing the passage of a number of different compounds (Contreras & Carrasco, 1979; Lacal et al., 1980). The fact that this change is not specific is reinforced by our findings that membrane potential dropped at around the fourth h after poliovirus infection. Our experiments with guanidine and cycloheximide indicate that viral gene expression is required to modify membrane permeability, in agreement with previous observations (Nair et al., 1979; Carrasco, 1981; Carrasco & Esteban, 1981). Both guanidine and cycloheximide prevented the development of membrane leakiness if they were present from the beginning of infection. However, if the virus was allowed to replicate for 1 or 2 h before adding guanidine, the permeability to 86Rb+ ions was altered. This result contrasts with that shown in Fig. 3, where addition of cycloheximide to block viral protein synthesis up to 2 h after infection led to no alteration of the membrane. Since guanidine slows down viral replication, but does not totally block the translation of the viral mRNAs present in the cytoplasm, a plausible explanation for these results could be that small amounts of viral proteins are involved in membrane alterations. These proteins would be synthesized even if guanidine was added 1 or 2 h after poliovirus infection, although this would not be the case in the presence of cycloheximide. From our experiments, we do not think that viral RNA itself is involved in this alteration since the addition of cycloheximide 2 h post-infection will allow its synthesis, because some viral replicase would have already been synthesized (Carrasco & Smith, 1980).

Poliovirus induces a drastic inhibition of cellular protein synthesis during the first 2 h of infection (Baltimore, 1969; Bablanian, 1975). At that time, there are no changes in the 86Rb+ content of the cell. This result suggests that the modification of monovalent ions in poliovirus-infected cells is not involved in the early shut-off. It still remains to be established whether other modifications in membrane permeability that occur very early during infection are connected with the inhibition of translation. Nevertheless, it is certain that the bulk of poliovirus protein synthesis takes place under ionic conditions quite different from those present in an uninfected cell, as occurs in other cells infected with picornaviruses (Egberts et al., 1977; Nair et al., 1979).

One of our conclusions has been that this alteration in membrane permeability correlates with the findings that poliovirus mRNA translation has developed the property of being resistant to high concentrations of monovalent ions (Saborio et al., 1974), as occurs in several viral systems which have been analysed (Nuss et al., 1975; Carrasco et al., 1979).

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