Cation Content in Poliovirus-infected HeLa Cells

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SUMMARY

Poliovirus-infected HeLa cells increased their permeability to monovalent ions from the third hour post-infection. At that time infected cells lost their content of potassium ions as measured by efflux of the potassium analogue $^{86}\text{Rb}^+$. The rate of release of $^{86}\text{Rb}^+$ from cells increased as infection proceeded, and was not sensitive to ouabain or quinidine, inhibitors of the $\text{Na}^+/$$\text{K}^+$ ATPase and $\text{Ca}^{2+}$-induced $\text{K}^+$ release system, respectively. The leakage of $^{86}\text{Rb}^+$ was only slightly sensitive to furosemide, an inhibitor of the $\text{Na}^+/$$\text{K}^+/$$\text{Cl}^-$ cotransport system, suggesting that the mechanism of release involved an increased passive permeability of the cell membrane. The calcium content or its efflux did not vary significantly in the infected cells. Neither were there alterations in the intracellular pH throughout infection.

INTRODUCTION

A number of animal viruses modify the plasma membrane during infection (Kohn, 1979; Carrasco & Lacal, 1984). For example, picornaviruses cause modifications in the permeability of the infected cells both during virus entry (Carrasco, 1981) and in the late phase when the bulk of viral components are being synthesized (Carrasco, 1978; Contreras & Carrasco, 1979). The early modifications in permeability are restricted to the entry of the virion particles together with several non-permeant compounds into the cytosol (Fernández-Puentes & Carrasco, 1980; Carrasco, 1981; Carrasco & Esteban, 1982). No redistribution of monovalent cations is observed at these early times, nor have changes been detected in membrane potential (Lacal & Carrasco, 1982). In contrast, drastic changes in permeability do take place in the late phase of infection. These modifications were first described by Farnham & Epstein (1963) who observed an increased entry of sodium and a leakage of potassium from the 3rd h after infection of L cells with encephalomyocarditis (EMC) virus. Similarly, EMC virus-infected 3T6 cells begin to lose their potassium content from the 4th h post-infection (Carrasco & Smith, 1976). Before such changes in cation concentrations are detected there is an increased permeability to non-permeant compounds, such as nucleotide analogues and several translation inhibitors (Lacal & Carrasco, 1982).

A parallel has been found between changes in permeability to monovalent cations and the shut-off of translation in EMC virus-infected cells (Carrasco & Smith, 1976; Lacal & Carrasco, 1982), whereas in poliovirus-infected cells the shut-off precedes the collapse of monovalent ion gradients maintained by the cell membrane (Nair, 1981; Lacal & Carrasco, 1982; Schaeffer et al., 1982). Moreover, guanidine, a selective inhibitor of poliovirus replication, does not prevent the shut-off of host protein synthesis, yet the cell membrane is able to maintain the distribution of monovalent cations (Nair et al., 1979; Lacal & Carrasco, 1983).

Recent studies by Koch and coworkers indicate that a modification in membrane potential occurs in poliovirus-infected HeLa cells. Also, the piretanide-sensitive $^{86}\text{Rb}^+$ uptake due to the $\text{Na}^+/$$\text{K}^+/$$2\text{Cl}^-$ cotransport system is inhibited from the 2nd h of poliovirus infection (Schaeffer et al., 1984).
These observations suggest that the early shut-off of host protein synthesis induced by poliovirus infection is not a direct consequence of changes in monovalent cations, although the possibility that a change in the membrane is involved cannot be discarded.

The aim of the present study was to determine the effects of viral infection on intracellular pH and the distribution of monovalent and divalent cations using poliovirus-infected HeLa cells.

**METHODS**

**Cells and virus.** HeLa cells and African green monkey kidney cells (Vero) were grown in Dulbecco's modified Eagle's medium (E4D) containing 10% calf serum (E4D10) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

Poliovirus type 1 (Mahoney strain) was grown in HeLa cells in E4D containing 2% calf serum (E4D2). Cells and medium were collected and sedimented at 4000 r.p.m. for 20 min in a bench-top centrifuge. The pellet was resuspended in distilled water, frozen and thawed three times, centrifuged at 4000 r.p.m. for 15 min and the supernatants of both centrifugations were mixed and used as the source of virus.

**Virus infection and determination of protein synthesis.** Cells were grown in 24-well Linbro plates containing 0.5 ml E4D10. Viral infection was carried out in 0.2 ml of E4D2 and after 1 h incubation at 37 °C, the medium was removed and fresh E4D2 medium was added. The time of virus addition was taken as -1 h and zero time was when the virus was removed. At the times indicated in Results the medium was aspirated, and the cells were washed once with methionine-free medium before incubation with 0.2 ml of methionine-free medium (E4D1) containing 0.1 μCi [³⁵S]methionine (600 Ci/mmol, Amersham). After 1 h incubation, the medium was removed and the cell monolayer was washed with phosphate-buffered saline and precipitated with 5% TCA. The pellet was then washed twice with ethanol and dissolved in 0.2 ml of 0.1 M NaOH containing 0.1% SDS. Radioactivity was determined in an Intertechnique scintillation spectrometer.

**Determination of ⁸⁶Rb⁺ content and ⁸⁶Rb⁺ fluxes.** Confluent cultures of HeLa cells grown in 24-well plates were incubated overnight in E4D2 medium with 1 μCi of ⁸⁶RbCl/ml. To determine ⁸⁶Rb⁺ content the radioactive medium was aspirated and the cultures and were quickly washed four times with 1 ml cold 0.1 M-MgCl₂. After the last wash, 200 μl of 5% TCA was added to the cultures to extract radioactivity. The uptake of ⁸⁶Rb⁺ was measured in 24-well plates after 15 min pulses with 200 μl of E4D2 containing 1 μCi of ⁸⁶Rb⁺/ml. The uptake was stopped by washing the cultures five times with 0.1 M-MgCl₂, pH 7.0 at 4 °C. The radioactivity was extracted with 200 μl of 5% TCA. When appropriate, 1 mM-ouabain was added during the uptake experiments. Ouabain-sensitive ⁸⁶Rb⁺ uptake was calculated by subtracting ⁸⁶Rb⁺ uptake in the presence of ouabain from ⁸⁶Rb⁺ uptake in the absence of the inhibitor.

**Efflux of ⁸⁶Rb⁺ from ⁸⁶Rb⁺-loaded cells.** Cells were incubated overnight in E4D2 medium in an atmosphere of 5% CO₂ and 95% air in 0.5 ml of E4D2 medium after removing the labelling medium and washing the cultures three times with 1 ml of E4D medium at 37 °C. At the times indicated in Results, samples of 50 μl were taken for scintillation counting and were replaced by 50 μl of fresh medium. The radioactivity of the cells at the end of the efflux was extracted with 1 ml of a solution containing 0.1% SDS and 0.1 M- NaOH. The sum of all the radioactivity in each sample plus the radioactivity left in the efflux period was taken as the total radioactivity present in the cells at the beginning of the experiment.

**Intracellular ⁴⁵Ca²⁺ content and efflux.** HeLa cells were loaded overnight with 4 μCi of ⁴⁵Ca⁺/ml in E4D2 medium. At the post-infection times indicated in Results the cultures were washed six times with 1 ml E4D medium containing 3 mM-EGTA and the radioactivity was extracted with 0.1% SDS in 0.1 M- NaOH.

The determination of ⁴⁵Ca⁺ efflux was carried out as described above for ⁸⁶Rb⁺.

**Intracellular pH.** Intracellular pH was measured with the weak acid 5,5-dimethyl-[2-¹⁴C]oxazolidine-2,4-dione (DMO) (Waddell & Butler, 1959). Briefly, cultures were washed twice with 2 ml of modified E4D2 medium (60 mM-NaCl, 45 mM-NaHCO₃, 38 mM-HEPES/Tris buffer, pH 7.0) and incubated at 37 °C with 1 ml of the same medium. At the times indicated in Results 40 μl of ¹⁴C-DMO (final concentration 126 μM, 1.5 × 10⁶ to 1.8 × 10⁶ c.p.m./dish) was added to the cultures. After 15 min incubation in humidified 5% CO₂/95% air at 37 °C, the medium was aspirated and the cultures were washed quickly (5 to 5.5 s per dish) by sequentially dipping the dish once in each of four beakers containing 200 ml of 0.1 M- MgCl₂, 10 mM-Tris-HCl pH 7.0 at 4 °C. The monolayers were then dried and the cells solubilized with 0.6 ml of 0.1 M- NaOH, 2% Na₂CO₃, 0.1% SDS. The radioactivity was measured using a liquid scintillation spectrometer. Protein was determined as described by Lowry et al. (1951).

Correction for DMO trapped in extracellular water was made by subtracting the radioactivity remaining in pre-cooled cultures washed immediately after addition of ¹⁴C-DMO. Each experimental value represents a mean of three replicates.

**Intracellular water.** Intracellular water was determined using ¹³C-urea. Urea distributes into total water space and can therefore be used for measurement of intracellular water space if the amount distributed in extracellular...
Cations in poliovirus-infected cells

fluid can be accounted for, or removed by washing. Extracellular [14C]urea was removed by five rapid washes of the cells with 5 ml of cold 0.1 M-MgCl₂, 10 mM-Tris-HCl pH 7. Negligible amounts of urea leaked out of the cells during the washing procedure.

For measurements of intracellular water, the cultures were incubated in 1 ml of modified E4D2 (see above) at 37 °C, and at the times indicated in Results were pulse-labelled with 40 μl of [14C]urea (final concentration 0.1 mM, 1.2 × 10⁶ c.p.m./dish). After 15 min at 37 °C, cultures were rapidly washed five times with 0.1 M-MgCl₂, 10 mM-Tris-HCl pH 7.0 at 4 °C. Trapped extracellular [14C]urea, which was not removed by washing, was estimated by rapid washing of pre-cooled dishes immediately after the addition of [14C]urea. These values were subtracted from total counts of the monolayers to obtain intracellular c.p.m.

RESULTS

Intracellular content, uptake and release of ⁸⁶Rb⁺ in poliovirus-infected HeLa cells

Infection of confluent cultures of HeLa cells with poliovirus type 1 at an m.o.i. of 50 pf.u./cell caused an early shut-off of cellular protein synthesis (Fig. 1). In agreement with previous findings (Nair, 1981; Lacal & Carrasco, 1982), late in poliovirus infection there was a marked decrease in intracellular potassium (measured as ⁸⁶Rb⁺) (Fig. 1). In an attempt to study the precise mechanism of this ⁸⁶Rb⁺ leakage in the infected culture, we analysed the various transport systems involved in the regulation of the intracellular levels of potassium. First, we determined the uptake of ⁸⁶Rb⁺ into the cells at various times during poliovirus infection (Fig. 2). The results indicated that, commencing at the 3rd h, there was an inhibition of the ouabain-
Fig. 3. Effect of poliovirus infection on the release of $^{86}$Rb$^+$ from $^{86}$Rb$^+$-loaded HeLa cells. Cultures of HeLa cells in 24-well plates were loaded with $^{86}$Rb$^+$ as described in Methods. (a) At the indicated times, control (●) and poliovirus-infected cultures (0 h, ○; 1 h, △; 2 h, □; 3 h, ●; 4 h, ▽; 5 h, ▲; 6 h, ◀) were washed four times with 1 ml of E4D at 37 °C and the release of $^{86}$Rb$^+$ into E4D2 medium at 37 °C was determined at various times during the following 60 min as described in Methods. (b) Cultures of HeLa cells were infected with poliovirus at an m.o.i. of 50 p.f.u./cell. Guanidine-HCl (3 mM) was added to the cultures at times corresponding to 0 h (●), 1 h (△), 2 h (□), 3 h (▽) or 4 h (▲) post-infection. Efflux of $^{86}$Rb$^+$ was determined over a 60 min period commencing at 4 h after infection, as described in Methods. (c) Four h after infection of $^{86}$Rb$^+$-loaded cultures of HeLa cells with poliovirus (○), efflux of $^{86}$Rb$^+$ into the medium was determined in the presence of 0.5 mM-furosemide (▽) or 0.1 mM-quinine (▽). All results are expressed as percentages of levels at time zero, i.e. 4 h post-infection.

inhibitable component of $^{86}$Rb$^+$ uptake. Simultaneously, the component of uptake of $^{86}$Rb$^+$ that was not mediated by the Na$^+$/K$^+$ pump was also inhibited. However, at an earlier stage of infection there was a significant increase in $^{86}$Rb$^+$ uptake through a ouabain-insensitive pathway (Fig. 2). This early increase in $^{86}$Rb$^+$ uptake was not inhibited by cycloheximide or guanidine (results not shown).

Since the plasma membrane of animal cells is impermeable to K$^+$, the inhibition of K$^+$ uptake systems alone could hardly explain the rapid decrease of $^{86}$Rb$^+$ levels that occurs in the late phase of infection. In order to ascertain whether infection with poliovirus enhanced membrane permeability, we measured its effect on the efflux of $^{86}$Rb$^+$ from $^{86}$Rb$^+$-loaded cells. Fig. 3(a) shows that $^{86}$Rb$^+$ leaked out from cells at an increased rate beginning at the 3rd h after infection. To determine whether the increased permeability was dependent on viral gene expression, we studied the effect of guanidine-HCl on the efflux of $^{86}$Rb$^+$ from pre-loaded cells (Fig. 3b). The presence of 3 mM-guanidine from the beginning of infection prevented the increase in membrane permeability as measured at 4 h, suggesting that poliovirus replication and viral gene expression are necessary events in the development of membrane leakiness. The same result was obtained when guanidine was added 1 or 2 h after poliovirus. However, addition of the inhibitor 3 h after infection did not fully prevent the modification of membrane permeability, even though the level of viral protein synthesis, analysed by gel electrophoresis, was greatly reduced (results not shown). These results suggest that the viral product(s) synthesized during the first 2 h of infection are not able to modify the permeability to K$^+$ ions and also that a rather small amount of viral product can induce a significant change in membrane permeability. The latter result was also confirmed in experiments carried out in HeLa cells pretreated with interferon (results not shown). In an attempt to understand the molecular basis of the altered K$^+$ permeability we used two inhibitors of K$^+$ transport systems, namely quinidine, which affects Ca$^{2+}$-induced K$^+$ release in various cell types (Gárdos, 1958; Reichstein & Rothstein, 1981), and furosemide, an inhibitor of the Na$^+$/K$^+$/2Cl$^-$ cotransport system (Gargus & Slayman, 1980). As shown in Fig. 3(c), only furosemide caused a slight inhibition of poliovirus-induced membrane permeability suggesting that the enhanced
permeability to $K^+$ in poliovirus-infected cells might reflect an alteration in the mechanisms controlling passive permeability (leakiness) rather than the modification of a specific $K^+$ transport system.

Intracellular pH is not altered in poliovirus-infected HeLa cells

Changes in the intracellular pH drastically affect various cellular functions (Roos & Boron, 1981). Furthermore the binding of different ligands to their cellular receptors is known to modify the intracellular pH through the activation of $H^+$-extruding systems present in the plasma membrane (Schuldiner & Rozengurt, 1982; L’Allemain et al., 1984).

In order to ascertain whether infection of HeLa cells with poliovirus type 1 could affect the intracellular pH, we measured this parameter throughout the infection by using the weak acid $[^{14}C]$DMO (Waddell & Butler, 1959). $[^{14}C]$DMO distribution between the extracellular medium and intracellularly depends on the pH gradient (Hirschie & Epel, 1981). The results shown in Fig. 4 indicated that, despite the drastic inhibition of protein synthesis, the intracellular pH remained unchanged. However, this result does not exclude the possibility that transitory changes in the intracellular pH may have occurred during infection.

Infection of HeLa cells with poliovirus type 1 does not change the cellular level of $^{45}Ca^{2+}$

Since calcium is a known mediator of many cellular processes (Cheung, 1983, 1984) and changes in calcium content have been proposed to be involved in the development of cytopathic effects in virus-infected cells (Trump et al., 1971; Schanne et al., 1979), we determined the intracellular $Ca^{2+}$ content of infected cells, using $^{45}Ca^{2+}$ as a tracer. The results shown in Fig. 5 indicated that infection of HeLa cells with poliovirus at a high multiplicity (m.o.i. 50) had no significant effect on the intracellular $^{45}Ca^{2+}$ level. Moreover, analysis of $^{45}Ca^{2+}$ efflux curves suggested that the intracellular distribution of $Ca^{2+}$ was not modified either (Fig. 6).
Fig. 5. Intracellular $^{45}\text{Ca}^{2+}$ content and protein synthesis in poliovirus-infected HeLa cells. Cultures of HeLa cells in 24-well plates were loaded with $^{45}\text{Ca}^{2+}$ as described in Methods. These cultures were infected with poliovirus type 1 (m.o.i. 50) and the intracellular $^{45}\text{Ca}^{2+}$ content (○) was determined at different times during the infection. Also shown are the levels of incorporation of $[^{35}\text{S}]$methionine (△) into TCA-precipitable proteins of parallel cultures. Results are expressed as percentages of control values.

Fig. 6. $^{45}\text{Ca}^{2+}$ distribution in poliovirus-infected HeLa cells. Cultures of HeLa cells loaded with $^{45}\text{Ca}^{2+}$ were infected with poliovirus (m.o.i. 50). Three h after infection the cultures were washed and the efflux of $^{45}\text{Ca}^{2+}$ into the medium was determined at various times as described in Methods. Results are expressed as percentages of initial (time 0) levels for control (○) and poliovirus-infected cells (●).

DISCUSSION

The alteration of the ionic milieu in animal cells influences a number of cellular functions (Boynton et al., 1982). Since animal viruses modify the concentration of monovalent cations in the cytoplasm of infected cells, it is of interest to quantify these changes so as to know more precisely when they occur. All of the picornaviruses studied so far alter the permeability of cellular membranes to monovalent cations (Carrasco, 1987). The changes observed in ionic content of mouse cells after infection with EMC virus parallels the inhibition of cellular translation, whereas no such parallel is observed for poliovirus-infected HeLa cells (Lacal & Carrasco, 1982). These cells start to lose potassium ions from around 3 h, probably due to a non-specific modification of membrane permeability together with the inhibition of the systems involved in the transport of this cation. Viral gene expression is necessary for these alterations to take place but it is not yet known which viral protein is involved. Possible candidates could be the viral proteins that are located on the cellular membrane (Semler et al., 1982), although their exact function remains to be determined. Since picornaviruses express the same proteins throughout infection it is reasonable to assume that the protein(s) involved in the modification of permeability to monovalent cations observed in the present study is synthesized from the very beginning of infection.

Presumably, this protein would start to accumulate in the cell membrane from the early stages of infection and gradually reaches threshold levels above which the ionic gradients can no longer be maintained. In our experiments the increased levels of synthesis of this protein seemed to occur after the 3rd h of infection and finally led to cell lysis. Interestingly, other proteins such as mellitin, thionins, etc., which are also known to lyse cells, have profound effects on cellular metabolism and permeability at concentrations below those needed to alter the distribution of monovalent ions (Carrasco, 1987). Thus, it would be interesting to investigate the possibility that the viral protein(s) involved in the above phenomenon might also affect cell metabolism when present in lower amounts.
The involvement of divalent cations, particularly calcium ions, in the regulation of physiological processes and cell function is well documented (Cheung, 1983, 1984). It has been suggested that calcium could play an important part in the shut-off of translation induced by viruses (Durham, 1978), but our attempts to find significant changes in Ca$^{2+}$ levels in infected cells have been unsuccessful. Neither cation content nor differences in $^{45}\text{Ca}^{2+}$ calcium fluxes were observed in poliovirus-infected cells. However, these results should not be taken as definitive evidence because it is still possible that, although total calcium content does not vary significantly, the distribution between free cytosolic calcium and that contained in intracellular stores may have been modified. Moreover, small differences in the concentration of calcium in the cytoplasm, not detected by our technique, could influence a number of cellular and viral functions.

The fact that the intracellular pH was not altered in poliovirus-infected HeLa cells, suggests that the regulation of this important parameter as measured with DMO remains constant throughout infection. This result does not exclude the possibility of transitory alterations.

In conclusion, we observe that the distribution of monovalent ions in poliovirus-infected cells varies at about the same time that the bulk of poliovirus translation takes place. The mechanism seems to involve a non-specific increase in permeability to monovalent ions and an inhibition of the transport systems for these ions. Neither the content of Ca$^{2+}$ nor the intracellular pH varies significantly during the first 3 h.

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