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# Enhanced Intracellular Calcium Concentration during Poliovirus Infection

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**The infection of human fibroblasts by poliovirus leads to a notable increase in the intracellular calcium concentration, $[Ca^{2+}]_i$, measured by microfluorimetry or by flow cytometry. $[Ca^{2+}]_i$ increases from 2 to 3 h postinfection, and by the fifth hour there is a 5- to 10-fold increase in $[Ca^{2+}]_i$. At this time postinfection there is active viral protein synthesis. The modifications in $[Ca^{2+}]_i$ are not observed in the presence of cycloheximide, guanidine, or Ro 09-0179, indicating that virus gene expression is required for the increase in $[Ca^{2+}]_i$. Attempts to identify the source of the intracellular $Ca^{2+}$ by using different inhibitors of calcium fluxes suggest that calcium enters from the culture medium through voltage-sensitive calcium channels.**

Calcium ions play a crucial role in regulating the vast majority of cellular processes (20). In the case of animal viruses, an increase in the concentration of this cation has been documented during the entry into cells of some enveloped animal viruses, such as Sendai virus (11) and influenza virus (12). Apart from these early modifications in calcium concentrations, there are profound changes in the distribution of this cation late during infection in virus-infected cells (3, 4). Thus, cytomegalovirus infection of human fibroblasts results in enhanced calcium entry into intracellular stores (18). This entry is blocked by verapamil, which suggests that calcium enters cells from the culture medium through calcium channels (18). The increased cellular calcium concentration could certainly play a part in the cytopathic effects induced by cytomegalovirus infection or in virus particle morphogenesis. This may also be the case for rotaviruses, because no cytopathic effect develops in calcium-deprived cultures (16, 24). Indeed, the cytopathic effects induced by rotavirus infection on host cells could be mediated by the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) triggered by the synthesis of a viral product (16). Attempts to identify this product point to protein NSP4 as being responsible for this effect (25). Infection of insect *Spodoptera frugiperda* cells with recombinant baculoviruses expressing individual rotavirus proteins showed a fivefold increase in calcium concentration upon expression of NSP4 but not with any of the other 10 rotavirus proteins (25). The exact mode of action by which NSP4 triggers increased calcium concentration remains to be elucidated.

In contrast to other animal virus-cell systems for which alterations in intracellular concentrations of calcium ions have been described, virtually nothing is known about changes in divalent cation concentrations in poliovirus-infected cells (3, 4). The use of radioactive $^{45}Ca^{2+}$ suggested no gross differences in the total amount of calcium between uninfected and poliovirus-infected HeLa cells (15). However, these studies did not provide clues as to the modifications in the concentration of cytoplasmic free calcium. The use of selective fluorescent probes gives a more accurate measurement of this parameter, using microscopy or flow cytometry (26). Therefore, we undertook a detailed analysis of the changes in calcium concentrations in poliovirus-infected HeLa cells by means of calcium binding probes.

Poliovirus type 1 (Mahoney strain) was propagated and titrated by plaque assay in HeLa cells. Cells were grown as monolayers at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Poliovirus infections were performed at a multiplicity of infection (MOI) of 50 PFU per cell in DMEM with 2% calf serum. After 1 h of adsorption, the medium was removed and the cells were placed in fresh DMEM–2% calf serum (hour 0 postinfection [p.i.]). Guanidine and verapamil were from Sigma, cycloheximide was purchased from Calbiochem, and Ro 09-0179 was a gift from H. Ishitsuka and K. Yokose (Nippon Roche Research Center, Kamakura, Japan). The probes fura-2 and fluo-3 were obtained from Molecular Probes in their acetoxymethyl ester (AM) forms. These compounds were dissolved in dry dimethyl sulfoxide and stored at −20°C until used. To estimate protein synthesis, HeLa cells grown in 24-well Linbro dishes were mock infected or infected with poliovirus. At the indicated times, the medium was replaced by 0.2 ml of methionine-free medium, and protein synthesis was estimated by the addition of 4 μCi of [$^{35}$S]methionine (1,220 Ci/mmol; Amersham). At the end of the labeling period the cells were washed and dissolved in 0.1 ml of sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol, 17% glycerol, 0.024% bromophenol blue as an indicator). Each sample was sonicated to reduce viscosity and heated to 90°C for 5 min. Ten microliters was applied to a 15% polyacrylamide gel and run overnight at 80 V. Fluorography of the gel was carried out, and the dried gels were exposed by using Agfa films at −70°C. Epifluorescence microscopy was carried out with fura-2 AM as a probe. HeLa cells were seeded onto coverslips (diameter, 2.5 cm; Thomas Scientific) and placed inside 35-mm-diameter culture dishes. One hour before measurements, the cells were washed and incubated with 4 μM fura-2 AM (1) in new DMEM containing 0.1% bovine serum albumin (Sigma) and 250 μM sulfinpyrazone (Sigma). After one hour of incubation at 37°C, the cells were washed twice with DMEM to eliminate the extracellular probe, and the coverslip was introduced into the measurement chamber. This special chamber was placed on the thermostatted stage of a fluorescent Zeiss Axiovert 35 M inverted micro-

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scope. Measurements were made at 37°C in different fields of the coverslips, focusing on small groups of cells. The ratio method was followed as described (27–29), using two excitement wavelengths, 340 and 380 nm, and one emission wavelength, 510 nm. $[Ca^{2+}]_i$ was obtained by using the equation $[Ca^{2+}]_i = K_d \times [(R - R_{min})/(R_{max} - R)] \times (F_{free}\,380/F_{sat}\,380)$, where $R_{min}$ and $R_{max}$ are the ratios obtained in 0 and 100% saturating calcium concentrations (22), respectively, and $R$ is the 340 nm-to-380 nm fluorescence ratio. We used 10 μM ionomycin plus 1 mM $CaCl_2$ to calculate the 100% saturation of the cytosolic probe ($R_{max}$) and 10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; pH 7.4] to obtain 0% saturation ($R_{min}$) (28, 29). $F_{free}380/F_{sat}380$ is the ratio of fluorescence intensities at these two extreme conditions (absence of calcium and saturating calcium concentration) for the wavelength 380 nm. $K_d$ is the effective fura 2-$Ca^{2+}$ dissociation constant and was taken to be 224 nM according to the work of Grynkiewicz et al. (8). Autofluorescence levels of the cells was calculated at the end of each run, by permeabilization with 1 ml of 160 mM digitonine in phosphate-buffered saline to wash the intracellular probe, using a perfusion pump. Cell autofluorescence levels at each excitation wavelength were subtracted to obtain actual measurements. Results were expressed as the arithmetical means of the measures taken from several groups of cells from five independent experiments. Flow cytometry analyses started with the detachment of mock-infected or poliovirus-infected HeLa cells from 35-mm-diameter dishes with trypsin (Sigma). Cells ($10^6$) were resuspended in DMEM–2% calf serum in Eppendorf tubes and incubated with 6 μM fluo-3 for 10 min at room temperature. Fluo-3, a long-wavelength calcium indicator, was chosen for cytometry studies because the emission (530 nm) and excitation (488 nm) characteristics make it better suited to flow cytometry analyses than fura-2 (Molecular Probes catalog). Fluo-3 is almost nonfluorescent unless bound to calcium. Thus, the fluorescence intensity of fluo-3 is higher in the presence of calcium ions. Propidium iodide (PI) (0.005%) was added, to estimate the proportion of dead cells. The PI-positive cells were always subtracted from all measurements. Samples containing $10^4$ cells were measured in a FACSCAN cytometer (Beckton Dickinson, San José, Calif.). The data were analyzed with Lysis II software (Becton Dickinson). In both cases (basal epifluorescence and flow cytometry), the background light levels of nonloaded cells were measured to determine the cell autofluorescence.

During poliovirus infection there are drastic modifications in the concentrations of intracellular monovalent cations, such that sodium ions enter into cells whereas potassium ions leak out (15, 17). Although changes in monovalent cation concentrations and pH during poliovirus infection of HeLa cells have been extensively analyzed (3, 4), virtually nothing is known about the potential modifications in the concentration of calcium ions. To this end, cells were loaded with fura-2 1 h before determination of the intracellular calcium concentration by microfluorimetry. Figure 1 shows that there is a clear enhancement in the intracellular calcium concentration from 2 to 3 h p.i. that reaches a 10-fold increase by the fourth hour p.i. The time when the concentration of calcium ions is enhanced in the cytoplasm of poliovirus-infected cells is coincident with the increase in membrane permeability to monovalent cations (15, 17). The kinetics of poliovirus protein synthesis in this experiment was analyzed in parallel each hour after infection. Figure 1B shows that viral proteins are clearly apparent from 2 to 3 h p.i., that synthesis is maximal at 3 to 4 h p.i., and that viral translation virtually disappears by the sixth hour p.i. Therefore,

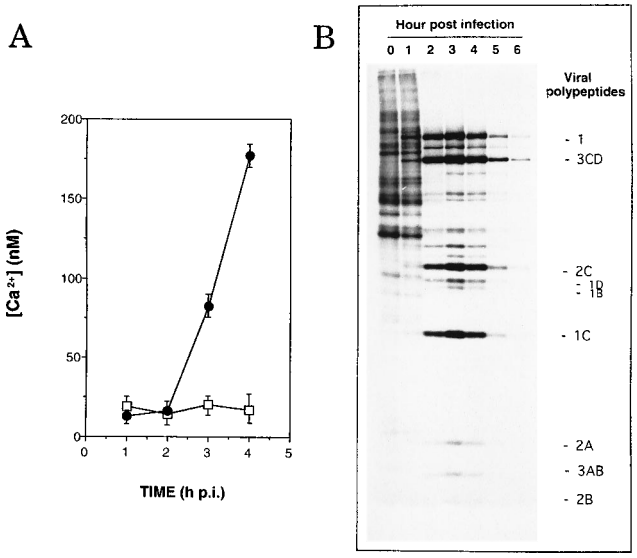


FIG. 1. (A) Determination of cytosolic free calcium concentrations during poliovirus infection, using microfluorometry; (B) kinetics of protein synthesis during poliovirus infection. (A) HeLa cells growing on 2.5-cm-diameter glass coverslips were infected with poliovirus at an MOI of 50 (closed symbols) or were mock infected (open symbols). One hour before each time indicated, cells were incubated with 4 μM fura-2 AM as described above. Measurements were performed using alternating dual-excition wavelength (340 nm and 380 nm) and recording emission at 510 nm. Data are means ± standard errors of measurements of different groups of cells from five independent experiments. (B) HeLa cells were infected at an MOI of 50. Proteins were labeled for 1 h with radioactive methionine at the indicated times, and equal volumes were analyzed by SDS-polyacrylamide gel electrophoresis. Viral proteins are indicated.

the translation of poliovirus mRNA takes place in cells with drastic alterations in cytoplasmic calcium concentration.

Once the concentrations of calcium ions were determined by microfluorimetry in the whole population of poliovirus-infected cells, we wanted to estimate the percentage of cells with these altered calcium concentrations. Thus, we made use of flow cytometry and the calcium probe fluo-3. HeLa cells infected with poliovirus were detached from the plate at the times indicated in Fig. 2A and incubated with fluo-3, and the percentage of cells with enhanced concentrations of calcium ions was estimated. Clearly, at the third hour p.i. about 30% of the cell population contains a higher intracellular calcium concentration, increasing to 70% by the next hour and virtually 90% by the fifth hour p.i. The fluorescence data in Fig. 2A indicate that there is about a 10-fold increase in the concentration of intracellular calcium in the population of cells that are not stained with PI. These results indicate that the calcium ion concentration increases during poliovirus infection of HeLa cells and that these changes take place at about the same time as do other modifications in membrane permeability (3, 4).

In order to test whether the changes in intracellular calcium concentration are a consequence of poliovirus gene expression, the infected HeLa cells were treated, just after poliovirus entry, with the inhibitor of protein synthesis cycloheximide or with one of two inhibitors of poliovirus genome replication, Ro 09-0179 and guanidine (2, 7). The intracellular calcium concentration was determined at 4 h p.i. (Fig. 2B). The three inhibitors block an increase in the calcium concentration in the infected cells, which clearly indicates that the inhibition of viral protein synthesis or poliovirus genome replication blocks the increase in cytosolic calcium concentration. The requirement

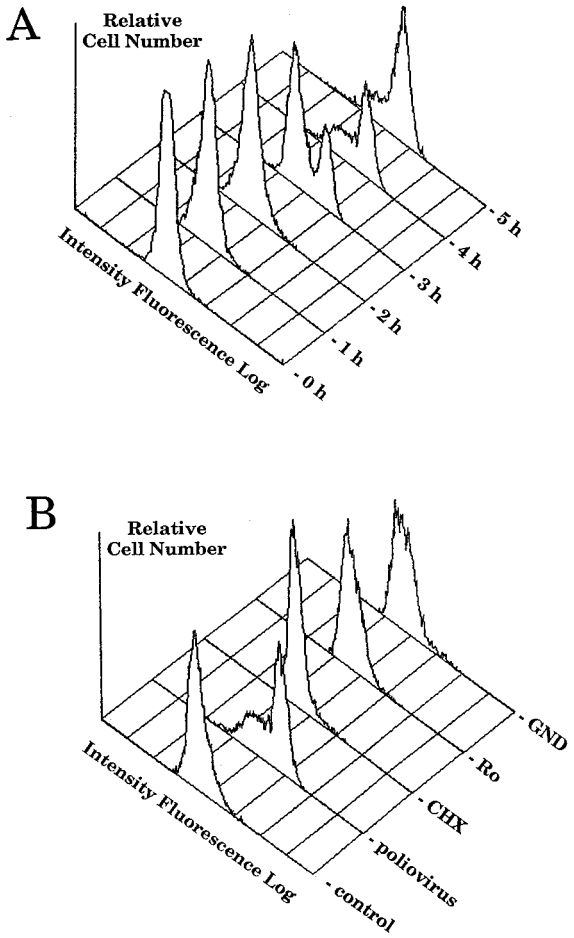


FIG. 2. Flow cytometry of calcium ions in poliovirus-infected HeLa cells. (A) Kinetics of cytosolic free calcium during poliovirus infection. Poliovirus infection was performed, as described above, at an MOI of 50 on cells growing in 35-mm-diameter culture dishes. Other cells were mock infected in parallel. At the indicated times p.i., mock-infected and poliovirus-infected cells were detached from plates, incubated with 6 μM fluo-3 (AM)–PI (0.005%), and analyzed in a FACSCAN cytometer. The intensities of fluorescence for infected cells are represented by a logarithmic scale; the values for mock-infected cells were identical at all times and were equal to the value at time zero for poliovirus-infected cells. The autofluorescence levels of cells and the PI-positive cells have been subtracted. (B) Effects of guanidine, cycloheximide, and Ro 09-0179 on cytosolic calcium concentration variations during poliovirus infection. Guanidine (GND) (500 μM), Ro 09-0179 (Ro) (1 μg/ml), or cycloheximide (CHX) (50 μM) was added after 1 h of poliovirus adsorption to mock-infected HeLa cells or to cells infected with poliovirus (MOI, 50). At 4 h p.i. cytosolic free calcium was monitored by flow cytometry. Also shown are the fluorescence intensities of both nontreated mock-infected cells (control) and poliovirus-infected cells.

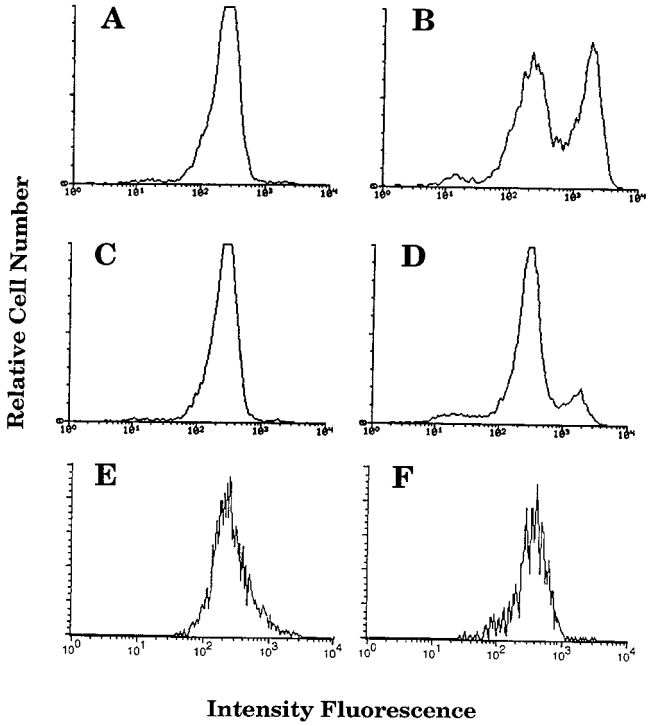


FIG. 3. Effect of verapamil and medium without calcium on $[Ca^{2+}]_i$. HeLa cells were mock infected or infected with poliovirus as described in Materials and Methods. After 1 h of adsorption and entry at 37°C, the virus inoculum was removed and the cells were placed in normal medium supplemented with 2% calf serum (A through D) or in calcium-free medium (E and F). For panels C and D cells were treated with 90 μM verapamil 2 h before flow cytometry, i.e., 2 h p.i. Flow cytometry was carried out at 4 h p.i. for nontreated mock-infected HeLa cells (A), nontreated poliovirus-infected HeLa cells (B), HeLa cells treated with 90 μM verapamil (C), and poliovirus-infected HeLa cells treated with 90 μM verapamil (D). After 1 h of adsorption time, mock-infected (E) or poliovirus-infected (F) cells were washed five times with calcium-free medium and incubated for 4 h in the same medium. Finally, flow cytometry was also performed in calcium-free medium. Quantitation of the two peaks that appear in panels B and D shows that 40% of the cells appear in the second peak in panel B, while 13% of the cells appear in the second peak in panel D.

for poliovirus genome replication in order to observe the increased $[Ca^{2+}]_i$ may be due to the need to produce enough viral proteins. In fact, there is a slight increase in the calcium concentration in cells treated with the inhibitors of viral RNA synthesis, but this increase is not significant. Therefore, the translation of the input viral RNA is not sufficient to promote the enhanced calcium concentration, at least during the first 4 h of infection. Nor can the changes be ascribed to alterations in the plasma membrane due to the entry of input virions.

The concentration of intracellular calcium ions can be raised by different mechanisms. One possibility is that pores are formed in the plasma membrane as a consequence of viral infection and extracellular calcium ions pass through these pores (3). Another possibility is that the increase in the cytoplasmic inositol triphosphate, produced by phospholipase C activation in poliovirus-infected cells (10), leads to a release of stored calcium from the endoplasmic reticulum during poliovirus infection. Treatment of cells with verapamil, an inhibitor of L-type voltage-gated calcium channels (6), reduces the cellular population with increased $[Ca^{2+}]_i$ in poliovirus-infected cells (Fig. 3), suggesting that at least part of the intracellular calcium comes from the external medium through voltage-sensitive calcium channels. Further support for this idea comes from the finding that poliovirus-infected HeLa cells placed in a medium without calcium do not undergo the increased $[Ca^{2+}]_i$ compared with the infected cells placed in normal medium (Fig. 3). The increase of $[Ca^{2+}]_i$ observed with calcium-free medium (about twofold) may indicate that a small proportion of the increase in cytoplasmic calcium concentration observed with poliovirus-infected cells comes from intracellular stores.

The finding that modifications in $[Ca^{2+}]_i$ occur in animal virus-infected cells raises at least three questions. (i) What is the mechanism of $[Ca^{2+}]_i$ increase? (ii) Which viral product(s) is involved? (iii) What cellular and viral functions are affected by the enhanced $[Ca^{2+}]_i$? With regard to the first question, it is possible that the increased calcium in poliovirus-infected cells comes from calcium influx through verapamil-sensitive

calcium channels. These channels could be formed by some poliovirus proteins that modify membrane permeability, e.g., proteins 2B and 3A (5, 14). In addition, it seems possible that increases in calcium concentration in animal virus-infected cells also occur as a consequence of phospholipase activation. Different animal viruses affect phospholipases to different extents (3, 19). Moreover, the same animal virus has different effects on phospholipase activity and, perhaps, membrane permeabilization, depending on the host cell. For instance, poliovirus infection greatly stimulates the activity of phospholipase C; and as a consequence, the production of choline, phosphorycholine, and the intracellular concentration of inositol triphosphate increases from the third hour of infection, while phospholipase A2 activity is inhibited in these cells (10, 13). Cells infected with Semliki Forest virus or vesicular stomatitis virus show increased activity of both phospholipases A2 and C (19). The finding that poliovirus-activated phospholipase C generated inositol triphosphate suggested that $[Ca^{2+}]_i$ might be augmented in the cytosol of infected HeLa cells (10, 13). Now, we provide evidence that, indeed, $[Ca^{2+}]_i$ increases in poliovirus-infected cells, but we still do not know if this increase is a consequence of binding to the inositol triphosphate receptor located in the endoplasmic reticulum. Our present studies indicate that the increased $[Ca^{2+}]_i$ mainly comes from the extracellular medium, although it is possible that a small percentage of the $[Ca^{2+}]_i$ increase comes from intracellular stores.

When considering changes in the intracellular ionic milieu in virus-infected cells, it is also important to elucidate the viral product involved. To conclude that a viral gene is responsible for these modifications, it should be demonstrated that a single gene product is able to elicit ionic changes when expressed individually in cells. In addition, viable virus mutants affected in that particular gene should be unable to modify the ionic concentrations after infection. To our knowledge, such data are not available for any animal virus. It may be that for at least some viruses such evidence could never be obtained, if mutant viruses in this gene are not viable. Attempts to individually express animal virus proteins to identify those responsible for modifying membrane permeability indicated that poliovirus proteins 2B and 3A (14), Semliki Forest virus 6,000-molecular-weight protein (6K protein) (23), and influenza virus M2 protein (9) are highly lytic for bacterial cells. Moreover, influenza virus M2 protein enhances the permeability to monovalent ions in *Xenopus* oocytes (18), while poliovirus protein 2B and, to a lesser extent, poliovirus protein 3A permeabilize mammalian cells to hygromycin B (5), indicating that membrane-active proteins from animal viruses act on both prokaryotic and eukaryotic membranes. The best example illustrating that expression of an individual viral protein increases $[Ca^{2+}]_i$ is provided by rotavirus protein NSP4. Expression of the nonstructural glycoprotein NSP4 increased $[Ca^{2+}]_i$ in insect Sf9 cells (26). This effect was not observed when the other 10 rotavirus proteins were expressed in these cells. Moreover, expression of this protein NSP4 was highly cytotoxic for cells, perhaps reflecting the activity of this protein on membrane permeability. However, no studies connecting NSP4 with changes in membrane permeability are yet available.

A generalized effect observed with compounds that kill cells is an increase in calcium concentration. Therefore, the idea that the enhanced $[Ca^{2+}]_i$ plays an important part in the virus-induced cytopathic effects and cell killing is widely accepted (3, 4). Thus, it has been hypothesized that NSP4 is directly involved in cytotoxicity and cell lysis induced by rotavirus infection (26). Apart from the effects of $[Ca^{2+}]_i$ on cell function, the increase in the concentration of this cation may also have consequences for virus replication. Calcium blockers, such as verapamil, are inhibitory for the replication of animal viruses, including human cytomegalovirus, Epstein-Barr virus, influenza virus, measles virus, and vaccinia virus (3). In the case of rotavirus, calcium plays a role in morphogenesis and is important for the compactness and integrity of virus particules (24). A generalized effect during the infection of susceptible cells by cytolytic viruses is the modification in monovalent ion concentrations (3). Since the concentration of cytoplasmic calcium governs a number of cellular processes, including the distribution of monovalent cations through the gating of monovalent ion channels, it could be that the modifications in monovalent cation concentrations in the infected cells are a consequence, at least in part, of the $[Ca^{2+}]_i$ increase. However, since there is a generalized increased membrane permeability in most animal virus-infected cells, not only to ions but also to low-molecular-weight compounds (3), it seems plausible to suggest that the opening of unspecific membrane pores by some virus proteins (3) is the consequence of the increased entry of monovalent and divalent cations in the infected cells. Additional studies aimed at the elucidation of the fluctuations of calcium ion concentrations will further our understanding of the cellular and viral functions influenced by modifications in calcium concentration during infection of cells by animal viruses.

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