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Mitochondrial Calcium Uptake During Infection of Chicken Embryo Cells with Semliki Forest Virus

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The key role of the mitochondria in the regulation of cellular Ca^{2+} led to a study of mitochondrial Ca^{2+} uptake during the infection of chicken embryo cells with Semliki Forest virus. Mitochondrial Ca^{2+} uptake was stimulated during the first 5 h of infection but declined later in infection. The early stimulation suggests an increase of cytoplasmic ionized Ca^{2+}, whereas the later decrease indicates mitochondrial injury. This functional deterioration was correlated with an increase of the permeability of the inner mitochondrial membrane. Polarographic experiments showed that electron transport is impaired, whereas transduction of energy to Ca^{2+} uptake is intact.

Among the most important functions of cellular membranes is the maintenance of ion gradients. The ionic composition of the intracellular milieu is an important factor in the control of metabolism (14). This makes it well worthwhile to ask whether the mechanisms regulating the intracellular ionic composition become changed under the influence of either cytolytic or transforming virus infections. Studies on cytolytic virus infections so far have been concerned with Na^+, K^+, and Mg^{2+} (9, 11, 16, 18).

Rather surprisingly, no information is available about the regulation of cellular Ca^{2+} during virus infection. This ion is known to be of key importance in metabolic control, and its role as a pleiotropic signal in the biology of the cell has become well established in recent years (6, 7, 31). The concentration of ionized Ca^{2+} in the cytoplasm is about 1 μM, whereas it exceeds 1 mM in the extracellular space. This considerable gradient is achieved by different factors (for a review, see 6): (i) by the relative impermeability of the cell membrane to Ca^{2+}; (ii) by an outwardly directed Ca^{2+} pump located at the cell membrane; (iii) by accumulation of Ca^{2+} from the cytoplasm by the mitochondria and the microsomes.

The mitochondria, therefore, play a dual role in the cell's regulation of this ion in both accumulating Ca^{2+} and, under aerobic conditions, supplying ATP required for the function of the Ca^{2+} pumps of the cell and microsomal membranes.

The present study quantitatively investigates the effect of a cytolytic virus infection on mitochondrial Ca^{2+} uptake. It shows that Semliki Forest virus (SFV) stimulates mitochondrial Ca^{2+} uptake early in infection, whereas the uptake of this ion decreases in the later stages. The mechanisms of the early stimulation and later decrease are discussed.

(Parts of this work were presented at the 9th Annual Meeting [1 April 1977] of the Union of Swiss Societies of Experimental Biology and were published in abstract form [E. Peterhans, E. Browse, and R. Wyler, Experientia **33**:827, 1977].)

MATERIALS AND METHODS

Cell culture. Primary chicken embryo cells (CEC) were prepared as described previously (30). A 100-ml amount of cell suspension (10^6 cells per ml) was seeded into a Roux flask (surface area, 215 cm^2) in medium 199 (Serva, Heidelberg, Germany) supplemented with 5% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), 100 IU of penicillin per ml, and 100 μg of streptomycin per ml.

Virus and infection of cultures. SFV, strain Kumba, was grown in the brains of 3-week-old ZUR:ICRZ mice obtained from the Institut fuer Zuchthygiene, University of Zurich. Three days after the intracerebral injection of about 10^3 PFU, mice were sacrificed and brains were homogenized in phosphate-buffered saline (PBS) containing 0.5% (wt/vol) bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, U.K.). Virus was purified by centrifugation at 750 × *g* for 15 min followed by sedimentation at 75,000 × *g* for 1.5 h. Virus was then resuspended at 4°C overnight, layered onto a 5 to 50% (wt/vol) potassium tartrate gradient, and centrifuged in an SW25 Spinco rotor for 2.5 h at 23,000 rpm. The resulting band of virus was diluted with PBS/bovine serum albumin, dialyzed against PBS, and titrated on CEC

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as described elsewhere (25), with an overlay containing Eagle minimum essential medium, 5% fetal calf serum, 0.1% DEAE-dextran, and 0.8% Noble agar. The cells of 48-h cultures were infected at a multiplicity of 20 PFU/cell. Controls were mock infected with PBS/bovine serum albumin. After adsorption of the virus for 90 min at 4 to 6°C, cells were washed with PBS and prewarmed fresh medium was added. Hours postinfection (p.i.) are calculated from the addition of fresh medium.

Polarographic procedures and measurement of mitochondrial Ca^{2+} uptake. Treatment of CEC with mannitol renders the cell membrane permeable to Ca^{2+}, and mitochondrial Ca^{2+} uptake can then be studied in situ. The experimental details of this technique have been described elsewhere (30). Briefly, the cells were detached from the supporting glass in mitochondria isolation buffer after they had been washed with the same buffer (0.27 M mannitol, 0.05% [wt/vol] bovine serum albumin, 0.1 mM EDTA, 10 mM Tris-hydrochloride, pH 7.3). After centrifugation at 600 × *g* for 7 min, the cell pellet was resuspended in a few drops of the same buffer. Protein concentration was determined by the biuret method (19). Cells were diluted into mitochondrial incubation buffer (0.25 M mannitol, 5 mM succinate, 10 mM KCl, 1.5 μM rotenone, 5 mM potassium phosphate, pH 7.2). The oxygen concentration of this buffer was determined, using sonically treated mitochondria and known amounts of reduced nicotinamide adenine dinucleotide, and was 485 ng-atoms of O per ml at 25°C. The protein concentration was 0.65 to 1.00 mg/ml (ca. 3.3×10^6 cells per mg of protein), and the final incubation volume was 0.95 to 1.00 ml. Respiration was stimulated by repeated addition of 125 nmol of $CaCl_2$. Changes of oxygen concentration were measured with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio), and traces were plotted with a W + W recorder, model 600 (Kontron, Zurich). Ca^{2+}/O quotients were calculated as described previously (17).

The $^{45}Ca^{2+}$ uptake by mitochondria was studied by using a rapid membrane filtration (Millipore Corp.) technique. Incubation was started by adding CEC (0.8 mg of protein) to 0.9 ml of incubation buffer containing, additionally, 1 mM $^{45}CaCl_2$ (ca. 300 cpm/ng-ion of Ca^{2+}) and 66 μg of digitonin per ml (30). The term "nanogram ion" of Ca^{2+} is used interchangeably with nanomoles of $CaCl_2$. Samples of 20 μl were taken at the times indicated, and the cells were retained on wet membrane filters (pore size, 0.45 μm; Millipore Corp.). Filters were quickly rinsed with 0.5 ml of ice-cold incubation buffer without $^{45}CaCl_2$, and the amount of $^{45}Ca^{2+}$ taken up was determined by liquid scintillation counting in a Packard liquid scintillation spectrometer, model 3375.

Permeability test for the study of mitochondrial injury. Cells were grown on microscope slides in plastic dishes of 5-cm diameter (Nunclon Plastics, Roskilde, Denmark). At 0, 2.5, 5, 7.5, 10, and 16 h p.i., the medium was aspirated from each of three control and three infected cultures, and cells were stained as described (1). The extent of staining was assessed with dark-field microscopy.

Trypan blue staining. Cells were grown in plastic dishes. Every 2 h p.i., the medium was aspirated from each of six control and six infected cultures. Cells were detached with 0.5 ml of 0.25% trypsin per dish, and 0.5 ml of cell culture medium was added to the cell suspension. One milliliter of 0.5% trypan blue was then added, and the percentage of stained cells was determined by counting 400 cells per dish.

Determination of cellular Ca^{2+} concentration. An identical specific activity of $^{45}Ca^{2+}$ was present in the media from the beginning of the cell culture until the determination of cellular $^{45}Ca^{2+}$ at different times p.i. Media were checked for total Ca^{2+} by atomic absorption and for $^{45}Ca^{2+}$ by liquid scintillation counting. At different times p.i., the medium was taken from each of four infected and four control cultures, and the cells were rapidly washed four times with cold PBS. Cells were then detached from the plastic dishes with a soft rubber policeman, and $^{45}Ca^{2+}$ was determined after solubilization of the cells in Rotiszint 22 (Roth Chemie, Karlsruhe, Germany). The detachment of cells by scraping was preferred to trypsinization because the latter treatment removes a significant percentage of cellular Ca^{2+} (3).

Determination of cellular Na^+ and K^+. Cells harvested for the measurement of mitochondrial Ca^{2+} uptake were prepared according to the method of Kotz et al. (23) for determination of Na^+ and K^+ in a Beckman atomic absorption spectrometer (type 1248).

Electron microscopy. At 5, 10, and 15 h p.i., infected and control cultures were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h. They were then washed in 0.1 M *s*-collidine buffer (pH 7.4), postfixed in 1% OsO_4 in this buffer for 30 min, briefly rewashed with *s*-collidine, detached from the glass dishes, and centrifuged at 140 × *g* for 15 min. The pellets were resuspended in 2.5% agar containing 0.1 M *s*-collidine (pH 7.4) at 60°C and centrifuged at 1,300 × *g* for 10 min. After cooling at 4°C, the pellets were cut into small blocks which were dehydrated in ethanol and embedded in Epon. From sections stained with uranyl acetate and lead citrate an equal number of photographs of each group was randomly taken, and the percentage of mitochondria showing pathological structure was determined. The classification was done according to Laiho and Trump (24), except that condensed mitochondria showing additional disruption of cristae were also rated as injured and no subdivision into various stages of degeneration was attempted.

RESULTS

Figure 1 shows $^{45}Ca^{2+}$ uptake by mannitol-treated cells during SFV infection. Both the rate and extent of $^{45}Ca^{2+}$ uptake are increased at the beginning (= 0 h p.i.) and at 2.5 and 5 h p.i., whereas they are essentially the same in infected and control cells at 7.5 h p.i. At 10 and 15 h p.i., $^{45}Ca^{2+}$ accumulation in infected cells is slower and less $^{45}Ca^{2+}$ is taken up as compared with controls.

Also, Ca^{2+} uptake by control cells increases p.i. The levels of Ca^{2+} accumulated by mock-infected cells at 10 and 15 h p.i. are comparable to those obtained early in infected cells. The

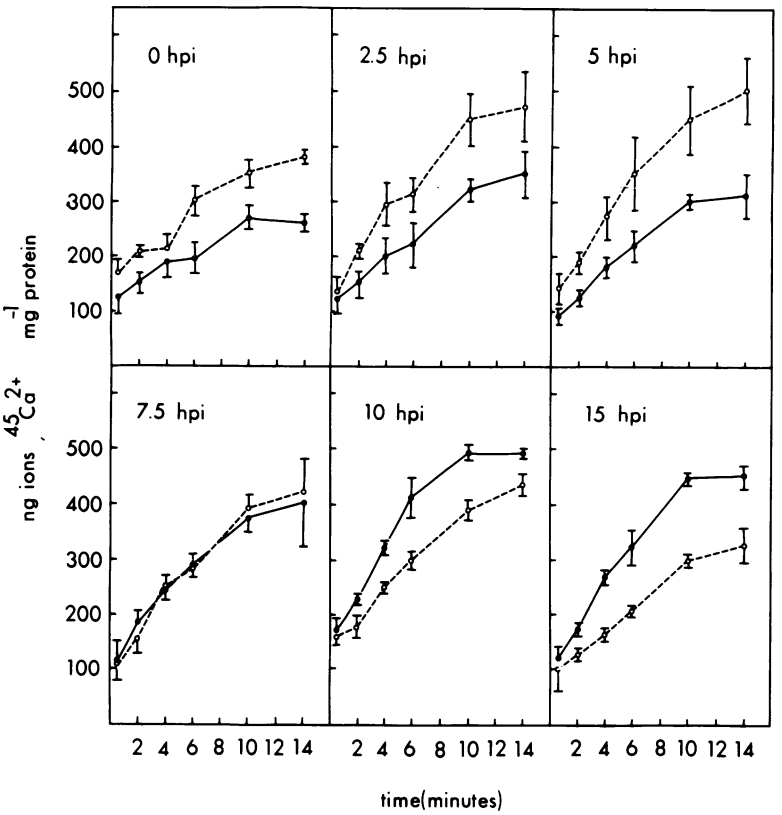

FIG. 1. *Mitochondrial $^{45}Ca^{2+}$ uptake during infection of CEC with SFV. $^{45}Ca^{2+}$ uptake measurement was started by adding mannitol-treated CEC to incubation buffer containing $^{45}Ca^{2+}$. Samples were taken, and unbound $^{45}Ca^{2+}$ was separated from the cells by filtration on 0.45-μm Millipore filters. The amount of $^{45}Ca^{2+}$ taken up is plotted versus incubation time in minutes. Time postinfection is indicated in hours (hpi). Bars indicate standard deviations of three successive incubations. Symbols:* (○) *infected;* (●) *control.*

$^{45}Ca^{2+}$ uptake increase in mock-infected cells may be a reaction to the medium change, given at the time of mock infection, and may be due to cell activation by serum. This phenomenon is treated in the Discussion.

Since several non-mitochondrial membrane systems (e.g., the cell membrane and the microsomes) are also able to bind or accumulate Ca^{2+} (6), we used ruthenium red as a specific inhibitor of mitochondrial Ca^{2+} transport (26) to assess the extent of non-mitochondrial Ca^{2+} binding and also to examine whether the differences between infected and control cells could be explained by a different degree of non-mitochondrial binding. As shown previously (30), $^{45}Ca^{2+}$ binding in the presence of ruthenium red was usually 10 to 15% of total $^{45}Ca^{2+}$ binding by mannitol-treated cells, and there was no significant difference between infected and control cells (not shown). This suggested that the differences observed were due to mitochondrial Ca^{2+} uptake. The high levels of Ca^{2+} accumulation by mannitol-treated cells (400 to 600 ng-ions of Ca^{2+} per mg of protein) contrast with the low Ca^{2+} levels found in intact cells (ca. 15 ng-ions of Ca^{2+} per mg of protein; see Fig. 4). This reflects the well-known potential of the mitochondria to accumulate large amounts of Ca^{2+} in the presence of phosphate and suitable substrates (7).

Decrease of mitochondrial Ca^{2+} uptake precedes gross cell membrane damage. Any functional impairment of a cell organelle makes it necessary to know whether this alteration precedes the lethal cell membrane damage occurring in all cytolytic virus infections. We chose two different approaches to investigate cell membrane function.

Since the cell membrane generates gradients for several ion species between the intra- and extracellular spaces, the determination of the cellular concentrations of some of these ions seemed to be a useful tool in assessing cell mem-

brane function. More indirectly, the extent of cell staining supposedly correlates with cell membrane damage.

Trypan blue staining of infected cells first increased between 10 and 12 h p.i. (Fig. 2), whereas cellular Ca^{2+} and Na^+ increased simultaneously with a drop of K^+, but not until later than 15 h p.i. (Fig. 3 and 4). This showed that cell membrane "damage," as assessed by the ability to sustain gradients of Na^+, K^+, and Ca^{2+}, occurred later than damage evidenced by trypan blue staining. It suggested also that the decrease in mitochondrial Ca^{2+} uptake, starting between 7.5 and 10 h p.i., was not a consequence of the breakdown of ionic gradients at the cell membrane, which did not occur until later than 15 h p.i.

Polarographic experiments. The decrease in mitochondrial Ca^{2+} uptake observed from 10 h p.i. on suggested that mitochondria had become functionally impaired during infection of CEC with SFV. To investigate the site of this functional impairment, we used a polarographic technique. The stoichiometry between O_2 consumption and Ca^{2+} uptake forms the basis of the functional characterization of mitochondria during Ca^{2+} accumulation. The substrates supplied in the incubation medium are transported into the mitochondria and dehydrogenated. Each pair of electrons passes through the electron transport chain, finally reducing 1 oxygen atom to water. The energy made available by this process can be used to either synthesize ATP or accumulate Ca^{2+}.

In the case of succinate (whose dehydrogenase is flavine adenine dinucleotide linked), four Ca^{2+} ions would be accumulated by fully coupled mitochondria per oxygen atom reduced. The Ca^{2+}/O quotient, then, in ideal mitochondria is 4 (calculated from the amount of Ca^{2+} added and oxygen used during the complete uptake of Ca^{2+}). Varying degrees of uncoupling of the transduction of energy to Ca^{2+} uptake lower this figure.

In control cells, the Ca^{2+}/O quotients were routinely 3.2 to 3.8, and oxygen consumption was 25 to 75 ng-atoms of O per mg of protein. By multiplication of Ca^{2+}-stimulated (= state 3) respiratory rate and the Ca^{2+}/O quotient, an estimate for the rate of Ca^{2+} uptake is obtained.

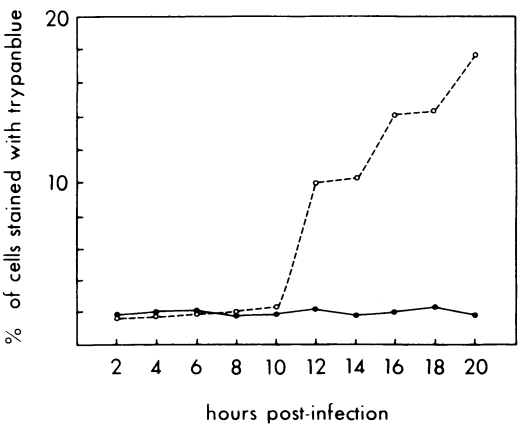

FIG. 2. *Trypan blue staining of CEC during infection with SFV. Every 2.5 h p.i., the percentage of trypan blue-stained cells was determined. The percentages represent the mean of each of six control and six infected cultures. In each culture, 400 cells were counted. Symbols:* (○) *infected;* (●) *control.*

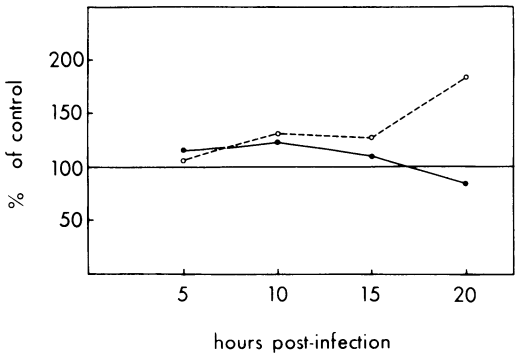

FIG. 3. *Cellular Na^+ and K^+ concentrations during infection of CEC with SFV. The cellular concentrations of K^+ and Na^+ were determined using atomic absorption spectrometry. Results, expressed as percentages of controls, represent the mean of three separate experiments and are corrected for the different protein contents of the samples. Symbols:* (○) *Na^+;* (●) *K^+.*

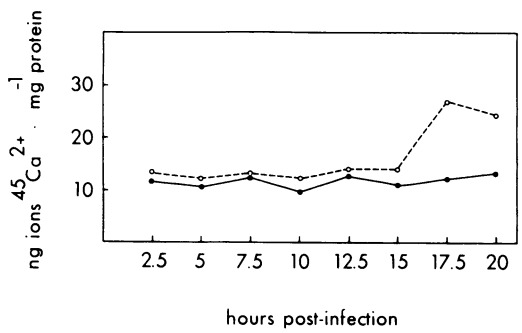

FIG. 4. *Cellular $^{45}Ca^{2+}$ concentration during infection of CEC with SFV. Cells were exposed to $^{45}Ca^{2+}$ of constant specific activity from the beginning of culture. At different times p.i., the amount of cellular $^{45}Ca^{2+}$ was determined using liquid scintillation counting. Results are expressed in nanogram ions of $^{45}Ca^{2+}$ per milligram of protein. Protein concentrations were determined from parallel nonlabeled cultures. Symbols:* (○) *infected;* (●) *control.*

The terms "state 3" and "state 4" respiration are used by analogy with the stimulated and resting respirations, respectively, obtained with ADP and are not meant to indicate identity between ADP- and Ca^{2+}-stimulated states.

Table 1 summarizes the polarographic experiments. The results are expressed in percentages of controls. The estimated rate of Ca^{2+} uptake correlates with the direct measurement of $^{45}Ca^{2+}$ uptake shown in Fig. 1, being increased at 5 h p.i. and decreased at 10 and 15 h p.i. The respiratory rates in the presence (= state 3) and absence (= state 4) of Ca^{2+} allow the nature of the decline to be determined. The decline could arise in either of two ways. First, viral infection could result in a progressive uncoupling of respiration, and, second, it could inhibit respiration. Uncoupling would result in a stimulation of state 4 respiration, whereas inhibition of respiration should decrease state 3 respiration. The state 3 respiratory rate declined noticeably p.i. (53% of controls at 15 h p.i.), whereas the state 4 respiratory rate was not stimulated (Table 1). Also, the Ca^{2+}/O quotient dropped, but at a slower rate than did the state 3 respiratory rate. Since the resting (state 4) respiration is present also during the uptake of Ca^{2+} (although not efficiently contributing to it [8]), the drop in the Ca^{2+}/O quotient reflected the simple fact that state 4 respiration contributed relatively more to total O_2 consumption when the stimulated respiration became lower.

Figure 5 shows traces from one polarographic experiment. It is obvious that even at 20 h p.i. the first addition of Ca^{2+} triggered a cycle of stimulated and resting respiration. Whereas state 3 respiration was considerably lowered in infected cells, relative to controls, state 4 respiration was similar. It can also be seen that the second addition of $CaCl_2$ slightly stimulated respiration but that the mitochondria failed to return to a state 4 respiration. This suggested that the mitochondria were in fact coupled, initially, but that they were uncoupled by higher amounts of Ca^{2+}. It can also be seen that although coupling in this experiment was rather poor at 5 h p.i., in both infected and control cell mitochondria, two cycles of state 3/state 4 respiration could be achieved in infected cells compared with only one in the controls. This is in qualitative accordance with infected-cell mitochondria taking up more $^{45}Ca^{2+}$ at 5 h p.i. than controls (Fig. 1).

TABLE 1. *Functional state of CEC mitochondria during infection with SFV*[a]

Time (h) p.i.	Ca^{2+}/O	State 3 respiratory rate	State 4 respiratory rate	(Ca^{2+}/O) × state 3 respiratory rate
5	105	109	104	114
10	91	79	87	71
15	73	53	104	39

[a] The functional state of the mitochondria was investigated by measuring O_2 consumption in an airtight vessel with continuous stirring. Respiration was stimulated repeatedly by injection of 125 nmol of $CaCl_2$ into the incubation medium containing 0.8 to 1.0 mg of cellular protein in a final volume of 1 ml. Results are expressed as percentages of controls and represent means of three cell batches infected separately. Ca^{2+}/O = Ca^{2+}/O quotient. State 3 respiratory rate = respiratory rate during the uptake of Ca^{2+}; state 4 respiratory rate = respiratory rate in the absence of Ca^{2+}; state 3 respiratory rate × (Ca^{2+}/O) = estimation of the rate of Ca^{2+} uptake.

Mitochondrial permeability test. The previous experiments suggested that the decrease in mitochondrial Ca^{2+} uptake during SFV infection of CEC was due to the inhibition of electron transport. The latter could be due to an unspecific alteration in the structure of the mitochondria or it could be the consequence of a more specific mechanism affecting respiration. An unspecific injury would increase the permeability of the mitochondria, whereas a specific inhibition would at least initially leave this parameter unchanged.

The principle of the method used to investigate either of these possibilities is the following: an intact inner mitochondrial membrane limits the rate at which *p*-nitroblue tetrazolium chloride and phenazine methosulfate reach succinate dehydrogenase located at the inner side of this membrane, whereas an increase in the permeability allows these reagents to reach the enzyme more rapidly (1). The enzyme reduces *p*-nitroblue tetrazolium chloride to a formazan, which can be viewed in dark-field microscopy as golden granules. No differences between infected and control cultures could be detected up to 7.5 h p.i. In contrast, from 10 h p.i. on, more granules were seen in infected cells (Fig. 6). The observation that SFV infection of CEC does not increase mitochondrial enzyme activity (12) supports the interpretation that this increased formazan formation reflects a permeability increase of the inner mitochondrial membrane.

Electron microscopy. Electron microscopy was undertaken to obtain further evidence that the decrease in mitochondrial Ca^{2+} uptake was correlated with alterations in the mitochondrial structure. Moreover, we wanted to know the approximate percentage of mitochondria showing morphological signs of injury. This should allow us to decide whether the decrease in mitochondrial Ca^{2+} uptake was mainly due to in-

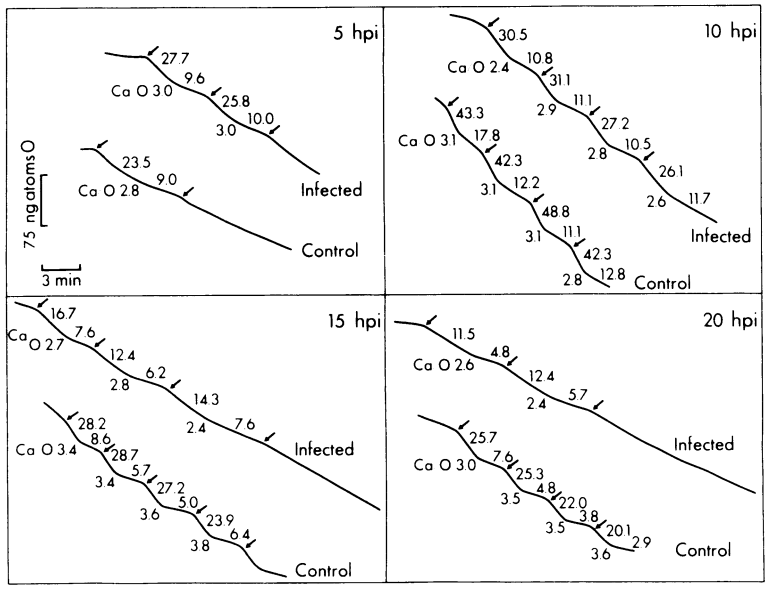

FIG. 5. *O_2 consumption of SFV-infected CEC during Ca^{2+} uptake. Cells were incubated at various times p.i. (hpi) in an airtight vessel under continuous stirring. Succinate at 5 mM was used as the substrate in the presence of 1.5 μM rotenone. Protein concentrations were 0.7 to 1.0 mg/ml and were identical in infected and control cells. Calcium uptake was started by injection of 125 nmol of $CaCl_2$ into the vessel and is marked by arrows. Respiratory rates are given in nanogram atoms of O per milligram of cellular protein. The final incubation volume was 0.95 ml.*

jury affecting most mitochondria simultaneously or whether it was correlated with a decrease in the fraction of mitochondria with intact structure. An equal number of photographs, randomly taken from infected and control cell preparations, was therefore screened, and the percentage of intact and injured mitochondria was determined.

The fraction of mitochondria showing completely intact structure remained constant in control cells, being 74 to 82%. In contrast, the percentage of mitochondria showing pathologically altered structure had increased to about twice the control level, leaving only 44 to 51% of the mitochondria intact in infected cells at 10 and 15 h p.i. The morphological changes were similar to those signs of mitochondrial injury described in the literature (24), ranging from condensation with rupture of cristae to swelling with complete loss of cristae.

Interestingly, the fraction of intact mitochondria was slightly (by 7%) less than controls already at 5 h p.i., when the mitochondria were still biochemically intact. This suggests that a quantitative correlation between the degree of morphological intactness and the functional state of a mitochondria population may not be reliable when dealing with minor morphological differences.

DISCUSSION

Impairment of membrane function is a well-known feature of cytolytic virus infection. The increased permeability of the cell and lysosomal membranes, resulting in the release of macromolecules from their respective compartments, has attracted much attention, although the role of lysosomal enzymes, in particular, in the pathogenesis of virus-induced cell damage is still controversial (2, 21, 29).

Much less is known about ion permeability of and transport by cellular membranes during cytolytic virus infection, probably because the regulatory effect of the ionic milieu on a wide variety of enzyme reactions has become fully appreciated only recently (14).

This is the first study reporting changes in one of the factors regulating the concentration of cytoplasmic Ca^{2+}. It shows that mitochondrial Ca^{2+} uptake is stimulated during the early stages of SFV infection of CEC, whereas it is impaired late in infection.

As to the latter, respiration was stimulated by Ca^{2+} even at 15 and 20 h p.i. and returned to a lower rate after the initial stimulation. However, whereas the resting rate was similar in infected and control cells, infection decreased the respiratory rate during the uptake of Ca^{2+} by about

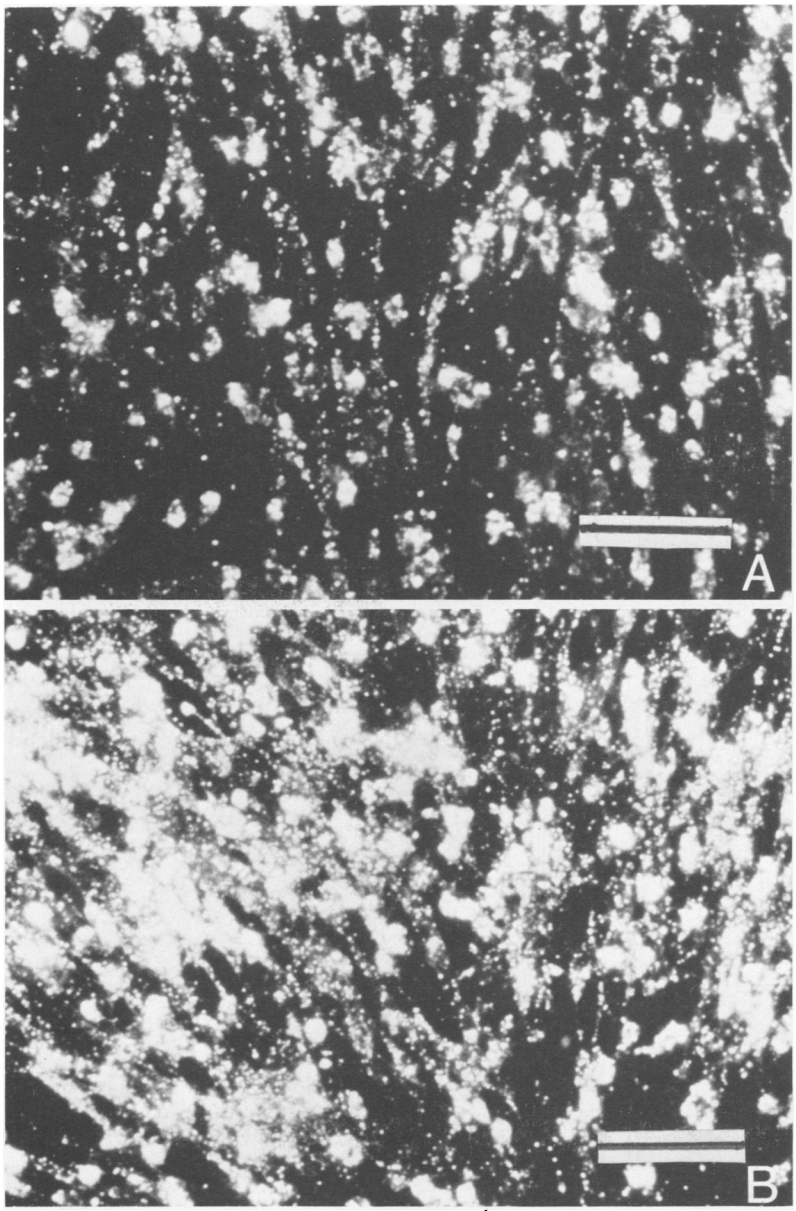

FIG. 6. *Mitochondrial permeability test in SFV-infected CEC. Cells were grown on microscope slides and stained for succinate dehydrogenase. (A) Control; (B) infected culture 10 h p.i. The infected culture shows more intense formazan formation, indicating an increase in the permeability of the inner mitochondrial membrane. Bars, 10 μm.*

50%. Taken together, these facts suggest that the decrease in mitochondrial Ca^{2+} uptake is due to an inhibition of electron transport rather than to uncoupling of respiration.

Concomitantly with the decrease in mitochondrial Ca^{2+} uptake, the permeability of the inner mitochondrial membrane for phenazine methosulfate and *p*-nitroblue tetrazolium chloride increased, as shown by the more intense formation of formazan in infected cells at 10 h p.i. (Fig. 6). This suggests that the functional deterioration is due to a nonspecific injury affecting the inner mitochondrial membrane rather than to a specific inhibition of electron transport. In sup-

port of this idea is the observation that simian virus 40 infection of green monkey kidney cells causes the release of enzymes from the mitochondrial matrix into the cytoplasm, concomitant with the decrease in O_2 consumption (28).

The decrease in mitochondrial Ca^{2+} uptake is correlated with a decrease in the percentage of mitochondria showing an intact structure. This suggests that there is no simultaneous injury affecting electron transport in all mitochondria. Rather, compartmentation seems to be important in this process, leaving the function of some mitochondria unaffected while damaging others to a higher degree than suggested by both polarographic and $^{45}Ca^{2+}$ uptake experiments.

The ion concentrations in infected and control cells may already be different early in infection, as suggested in Fig. 3 and 4 as well as by the stimulation of mitochondrial Ca^{2+} uptake shown in Fig. 1 (see below). The functional impairment of the mitochondria, however, is not correlated with such ionic alterations, which can be expected to occur with the breakdown of the gradients of Na^+, K^+, and Ca^{2+}. Furthermore, the lack of correlation between cell death as indicated by trypan blue staining and cell death as indicated by the breakdown of these ionic gradients between the intra- and extracellular spaces provides further evidence for the idea that the staining reaction reflects an ill-defined increase of the permeability of the cell membrane for the dye rather than metabolic death of the cell (28).

A stimulation of mitochondrial Ca^{2+} uptake early in the infection was also observed in a rabbit poxvirus-HeLa cell system (unpublished data). This alteration of mitochondrial Ca^{2+} transport may therefore be a more general feature of cytolytic virus infection.

Mitochondrial Ca^{2+} uptake also increased in controls, the levels reached late (10 and 15 h) after mock infection being similar to the early virus-induced changes (Fig. 1). This suggested that some constituent(s) of the medium was able to induce a change in mitochondrial Ca^{2+} uptake similar to the virus infection but that the time course was different.

Several growth-promoting agents such as serum and insulin stimulate glycolysis. This effect, also achieved by the Ca^{2+} ionophore A 23187, is believed to be mediated by an increase in cytoplasmic, free Ca^{2+} (13, 32, 33). A stimulation of glycolysis has been reported also in cells infected with SFV and other viruses (12, 22). Moreover, mitochondrial Ca^{2+} transport has been shown to be correlated with the extramitochondrial concentration of free (ionized) Ca^{2+}, being stimulated by rising Ca^{2+} (5). With this in mind, we treated CEC for 5 h with 0.5 and 5% fetal calf serum, respectively, and determined mitochondrial Ca^{2+} uptake at the end of this period. Pretreatment of CEC with a high concentration of serum caused a stimulation of mitochondrial Ca^{2+} uptake similar to that caused by virus infection (Fig. 7). Although time course experiments were not done in this system, the experiment suggests that the increase in mitochondrial Ca^{2+} uptake after mock infection could be due to the serum included in the cell culture medium. Serum at a concentration of 1% (vol/vol) had been used because the virus, in our hands, grew poorly in the absence of serum.

Based on the similar stimulation by serum and SFV of both glycolysis and mitochondrial Ca^{2+} uptake, a working hypothesis can then be formed, postulating that the virus induces an increase in cytoplasmic Ca^{2+}. Such a change could be due to an increase in the permeability of the cell membrane, leading to an increased influx of small ions such as Ca^{2+} from the extracellular space into the cytoplasm (9–11, 15, 16). On the other hand, Ca^{2+} could also increase by a release from intracellular stores such as the microsomes or the mitochondria.

The latter, in particular, cannot be ruled out, because Ca^{2+} may be transported into and out

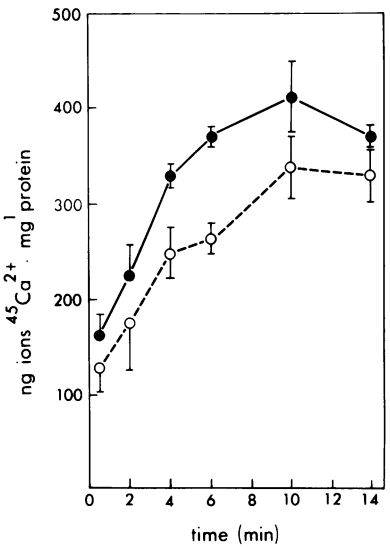

FIG. 7. *Effect of serum pretreatment on mitochondrial $^{45}Ca^{2+}$ uptake. CEC cultures (48 h old) were incubated for 5 h with medium containing 0.5 and 5% fetal calf serum, respectively. At the end of this period, mitochondrial Ca^{2+} uptake was determined, using $^{45}Ca^{2+}$ and Millipore filtration as in Fig. 1. The amount of $^{45}Ca^{2+}$ taken up is plotted against incubation time after the addition of Ca^{2+}. Symbols: (●) 5% serum; (○) 0.5% serum.*

of the mitochondria by different mechanisms (6, 7). A detailed kinetic analysis of subcellular Ca^{2+} fluxes, using isolated microsomes and mitochondria, as well as an analysis of Ca^{2+} fluxes through the cell membrane are necessary to decide between these possibilities.

If infection with SFV does indeed induce an increase in cytoplasmic Ca^{2+}, the early action of the virus may be very similar to that of serum and insulin. The final outcome of the interaction of these growth-promoting agents is quite different from virus infection, being cell proliferation as opposed to cell death in the case of the virus. The idea, however, that some stages of virus replication may require an activated state of the host cell is supported by observations made in lymphocytes. Lymphocytes activated by mitogens are more permissive for a wide variety of RNA and DNA viruses (34, 35), and the treatment of murine spleen cells with mitogens has been shown to cause the activation of endogenous xenotropic type C viruses (20, 27). Finally, influenza A viruses of the H_2N_2 subtype have been shown to be lymphocyte mitogens (4).

The well-known role of Ca^{2+} in cell activation, the effect of the activated state on virus growth in lymphocytes, and the observations reported in this paper suggest a role for Ca^{2+} in virus infection.

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