

Semliki Forest Virus-induced Polykaryocyte Formation is an ATP-dependent Event

By

C. KEMPF, URSULA KOHLER, M. R. MICHEL, and H. KOBLET

Institute for Hygiene and Medical Microbiology, University of Bern,
Bern, Switzerland

With 6 Figures

Accepted December 5, 1986

Summary

Infection of *Aedes albopictus* cells with Semliki Forest virus (SFV) leads to polykaryocyte formation below pH 6.2. This syncytium formation is accompanied by a decrease of the cellular ATP level. Addition of inhibitors of oxidative phosphorylation leads to a rapid, total depletion of ATP in infected cells at pH 6 and results in an inhibition of polykaryocyte formation. However, when cells were exposed for only a few minutes to pH 6 in the presence of the inhibitors and then kept at pH 7.2, the ATP level partially recovered to values sufficient for syncytium formation. Similar results were obtained after ATP depletion induced by 2-deoxyglucose. Thus, it can be concluded that SFV-induced syncytium formation is an ATP-dependent event.

Introduction

One of the most dramatic forms of membrane-membrane interaction is the fusion of cell membranes (for review, see reference 15), a phenomenon which occurs naturally both within cells, as required for cell division, and between cells (e.g., during myogenesis). Cell fusion is also a characteristic feature of certain tumors (5, 18) and viral infections (3, 6, 16). Membrane fusion occurs at the subcellular level during such events as endocytosis, receptor recycling, and delivery of endogenously synthesized proteins to appropriate subcellular destinations (1, 4, 17). Despite the biological importance of membrane fusion, very little is known about either the precise biochemical and biophysical events in fusion or the way in which cells control fusion of their membranes.

Several enveloped viruses have been reported to cause fusion under certain conditions (12, 14, 19). It was reported that *Aedes albopictus* cells, infected with Semliki Forest virus (SFV), an enveloped, positive-stranded RNA virus, undergo cell-cell fusion from within at mildly acidic pH, leading to polykaryocytes (11). KOBLET and collaborators reported that the initial step of this fusion was triggered by a conformational change of a surface protein, most probably a virus-coded protein (10). The syncytium formation could be inhibited by a wide variety of chemicals (e.g., ionophores, anesthetics, protein-modifying reagents, sodium azide), strongly suggesting the involvement of cellular factors in the control of the fusion event.

So far, these factors controlling fusion from within are unidentified and it is not clear how they are interconnected. Some metabolic reactions might be a prerequisite for fusion and others may be activated or inhibited as a consequence of fusion. Based on the finding that sodium azide and potassium cyanide—both potent inhibitors of oxidative phosphorylation—prevent fusion of SFV-infected *Aedes* cells, we investigated intracellular ATP levels during syncytium formation. In this report, we show that polykaryocyte formation in SFV-infected *Aedes* cells depends on the intracellular ATP level.

Materials and Methods

Chemicals

2,4-dinitrophenol and 2-deoxyglucose were purchased from Fluka, Switzerland. All other chemicals used were of the highest purity commercially available.

Cells and Virus

Aedes albopictus cells (clone C 6/36) (8) were cultured at 28°C in Mitsuhashi-Maramorosch medium (MM-medium) supplemented with 20 per cent fetal calf serum containing 100 µg of both streptomycin and penicillin per ml. The cells were passaged weekly by 1 : 10 dilutions. Semliki Forest virus (SFV) was propagated in *Aedes* cells. Virus titers were determined by a plaque assay according to established methods. All infections were carried out with a MOI of 10.

Fusion Assay

The pH of the medium in SFV infected cells was lowered at 16 hours p.i. from 7 to 6 for 1 minute or longer (10) as indicated for the different experiments. The polykaryocyte formation was assessed by means of light microscopy 30 minutes after the lowering of the pH if not otherwise stated. Fusion was considered to be positive when more than 90 per cent of the cells were integrated into the syncytium.

Determination of ATP Levels

Aedes albopictus cells were seeded into 24 well dishes (Costar) and grown to confluency. The monolayers (2 cm², 2 × 10⁵ cells/cm²) were either mock infected or infected with SFV. 16 hours p.i. the medium was removed by aspiration; the cells were washed with phosphate-buffered saline adapted for *Aedes* cells (PBS: 0.188 M NaCl, 3.35 mM KCl, 8 mM Na₂HPO₄, adjusted to pH 7.2) and incubated with the different drugs as described in detail in the result section. Then the PBS was removed and the cell monolayer solubilized in 1 ml 1 per cent Triton X-100. The amount of ATP was determined immediately with the luci-

ferin-luciferase system, purchased from Boehringer Mannheim, Federal Republic of Germany. Briefly, 100 μ l of Triton extract were added to 400 μ l of the reagent solution composed of 40 mM Hepes buffer pH 7.75, 4 mM EDTA, 0.36 mM dithiothreitol, 0.3 mM AMP, 20 mM $MgCl_2$, 0.7 mM D-luciferin and 1.6 μ g/ml luciferase from *Photinus pyralis*. The bioluminescence was measured in a Packard liquid scintillation counter, without the coincidence setting. ATP calibration solutions were prepared freshly before measurement by diluting a 5 mM stock solution with freshly quartz-distilled water containing 1 per cent Triton X-100, or MM-medium containing 1 per cent Triton X-100.

Results

Cellular ATP Concentration During Cell-Cell Fusion

When ATP standard curves were determined in presence of Triton X-100 and phosphate-buffered saline (PBS) or MM-medium they were linear in the range of 10^{-7} to 10^{-10} molar ATP. The standard curves showed a slope of $2.39 \times 10^{12} \pm 3.74 \times 10^{10}$ counts per second (cps), an ordinate interception of $4.12 \times 10^3 \pm 1.44 \times 10^3$ cps and a correlation coefficient of 0.999. ATP measurements in mock-infected cells at pH 7.2 or pH 6 showed a constant intracellular ATP concentration over the time periods investigated (Fig. 1). The cellular ATP level was identical in SFV-infected cells at pH 7.2 (Fig. 1) and was estimated to be in the range of 1–3 mM assuming a mean cell diameter of approximately 10 μ m. This value corresponds to normal intracellular ATP concentrations as described for other cell types. When *Aedes* cells were infected with SFV and the pH was lowered to 6 at 16 hours post infection (p.i.), fusion conditions were established, and the intracellular ATP level started to decrease over 60 minutes reaching 10–15 per cent of the initial value (Fig. 1). Concomitantly, the cells underwent fusion and the syncytium formation was completed after approximately 30 minutes. This decrease of intracellular ATP was not due to a leakage of the plasma membrane and diffusion of ATP into the extra-cellular space, since no increase in extracellular ATP could be detected. ATP added to MM-medium or PBS containing 25 mM glucose, also in low concentrations, is stable over one hour. This is in agreement with results of KOBLET and collaborators which also showed that SFV-induced cell-cell fusion of *Aedes albopictus* cells does not lead to a leakiness of the plasma membrane (unpublished observations). In contrast, mock-infected cells, when exposed to MM-medium of pH 6 or PBS pH 6 containing 25 mM glucose, showed a constant cellular ATP level (Fig. 1), identical to that of infected or mock-infected cells at pH 7.2. Thus, it can be concluded that the formation of polykaryocytes correlates with a decrease in intracellular ATP. However, this does not prove that ATP is used for fusion per se. The decrease in ATP could be due to the fact that the experimental conditions produce a large, artificial proton gradient across the plasma membrane so that ATP is used to maintain the intracellular pH at its physiological value. Therefore, additional experiments were per-

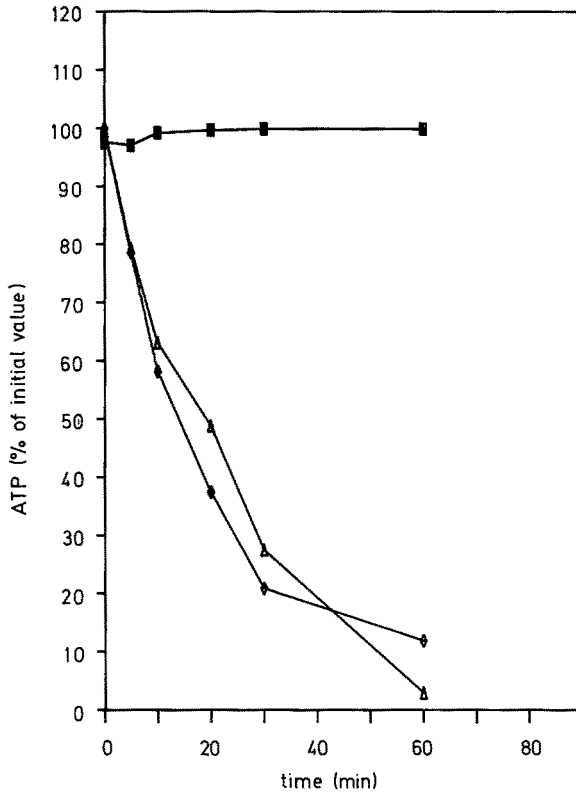


Fig. 1. *Aedes* cells were infected with SFV. 16 hours p.i. the growth medium was replaced by PBS pH 6 containing 25 mM glucose and the cellular ATP level was recorded over a period of 1 hour (—Δ—). Alternatively, SFV-infected cells were exposed for 1 minute to PBS pH 6 containing 25 mM glucose and then the PBS was replaced by either growth medium or PBS/glucose of pH 7.2 (—◇—). Determination of the ATP concentration in mock-infected cells at pH 7.2 or 6 (—□—) provided results identical to those of SFV-infected cells at pH 7.2

formed, where the external pH was restored to its original value. SFV-infected cells were exposed for 1 minute to pH 6 at 16 hours p.i.; then the pH 6 medium was replaced by growth medium or PBS, containing 25 mM glucose, of pH 7.2. These conditions also lead to polykaryocyte formation in case of infected *Aedes* cells (10). After a 1 minute exposure to pH 6, the syncytium formation was again accompanied by a decrease of cellular ATP, similar to the previous experiment, as depicted in Fig. 1. These results suggest that consumption of ATP during polykaryocyte formation is not due to the implied pH gradient across the plasma membrane. The finding that the ATP level also drops during fusion at pH 7.2, when the stress of an approximately 10 times higher H^+ concentration is released, is a good indication that formation of giant polynuclear cells is a process requiring ATP. This in turn would explain the finding mentioned above that NaN_3

inhibited cell-cell fusion. Therefore, we investigated several inhibitors of oxidative phosphorylation and used them to correlate the cellular ATP level with the syncytium formation.

*Influence of Inhibitors of Oxidative Phosphorylation
on Polykaryocyte Formation*

Aedes albopictus cells were infected with SFV. 16 hours after infection, the medium was replaced by PBS pH 6, containing 25 mM glucose and either 1 mM KCN, 1 mM NaN_3 or 10 μM dinitrophenol. Under these conditions, the infected cells were rapidly and totally depleted of ATP (Fig. 2) and polykaryocyte formation was prevented. These results further support the assumption that syncytium formation induced by low pH in SFV-infected cells requires ATP. Since depletion of cellular ATP by the various drugs mentioned above evidently occurs at a much slower rate at physiological pH, infected cells were not left at pH 6, but only exposed for 1 to 10 minutes to

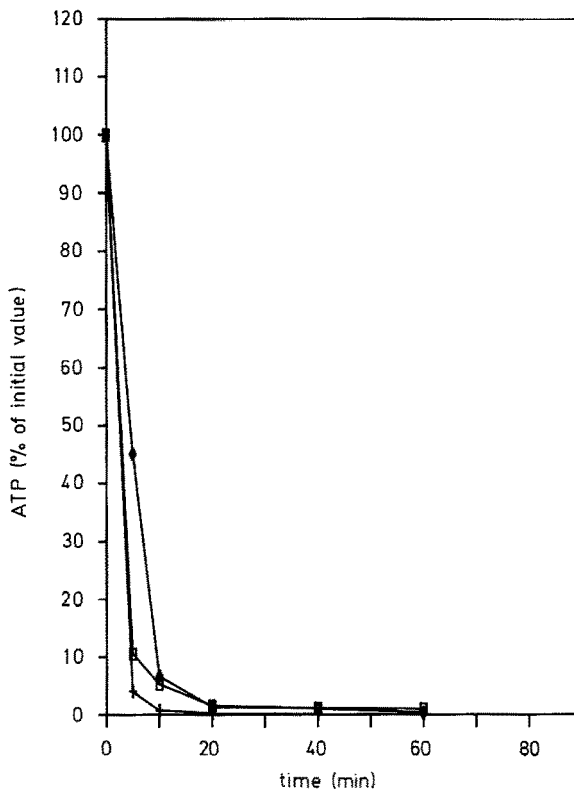


Fig. 2. 16 hours p.i. the growth medium was exchanged for PBS, pH 6, containing 25 mM glucose and either 10 μM dinitrophenol (-+-), 1 mM NaN_3 (-◇-) or 1 mM KCN (-□-), respectively. Cellular ATP was determined during 1 hour after the change of the medium.

Polykaryocyte formation was abolished by all the three drugs

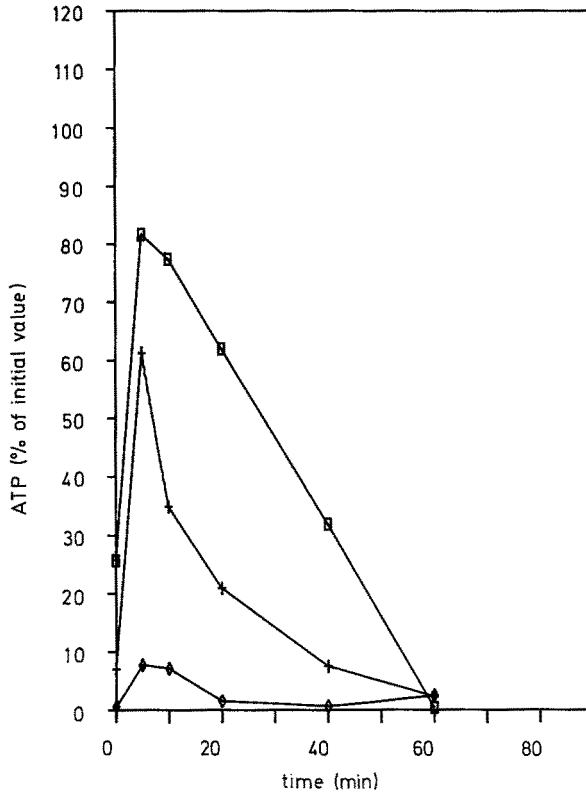


Fig. 3. SFV-infected *Aedes* cells were treated for 1 minute (—□—), 5 minutes (—+—), or 10 minutes (—◆—) with PBS/glucose, pH 6, containing $10\ \mu\text{M}$ dinitrophenol. Then this medium was replaced by PBS/glucose, pH 7.2, containing the inhibitor and thereafter cellular ATP was measured. After 30 minutes, the formation of giant polynuclear cells was observed in the experiments with a 1 and 5 minutes exposure to low pH, whereas a 10 minutes exposure to pH 6 in presence of dinitrophenol abolished syncytium formation.

pH 6 in presence of $10\ \mu\text{M}$ dinitrophenol to achieve a partial ATP depletion. Cellular ATP concentrations were recorded at regular intervals after restoring the pH to 7.2 and polykaryocyte formation was examined 30 minutes after the low pH exposure. As demonstrated in Fig. 3, this treatment leads to a rapid depletion of the cellular ATP as expected from the previous experiments shown in Fig. 2. However, replacement of the pH 6 medium after 1 minute with PBS of pH 7.2, containing $25\ \text{mM}$ glucose and the inhibitor, or with MM-medium of pH 7.2 and the inhibitor, resulted in a rapid, partial restoration of the intracellular ATP concentration before it started to decrease again. In this situation, polykaryocyte formation took place. When the cells were exposed for 5 minutes to pH 6 in presence of $10\ \mu\text{M}$ dinitrophenol, the recovery of the cellular ATP occurred to a lesser degree. Nevertheless, syncytium formation was still observed. Finally, a prolonged exposure of 10 minutes or more to the inhibitor and pH 6 led to an almost

total depletion of the cellular ATP level with only a slight restoration—less than 10 per cent—and the absence of polykaryocyte formation. Therefore, the results strongly suggest that fusion of SFV-infected cells triggered by low pH requires ATP and thus can be blocked by depletion of cellular ATP by inhibitors of the oxidative phosphorylation. If this assumption is correct, the other inhibitors of oxidative phosphorylation should show identical effects on intracellular ATP concentration and polykaryocyte formation under the conditions mentioned so far.

To this end, identical experiments were carried out in presence of 1 mM KCN. As depicted in Fig. 4, a 1 minute exposure to pH 6 in presence of the inhibitor followed by incubation at pH 7.2 resulted in a partial recovery of the cellular ATP level. This restoration was mainly expressed as an arrest of the decrease of cellular ATP over approximately 15 minutes—at 40 to 50 per cent of the initial value—accompanied by the formation of polykaryocytes.

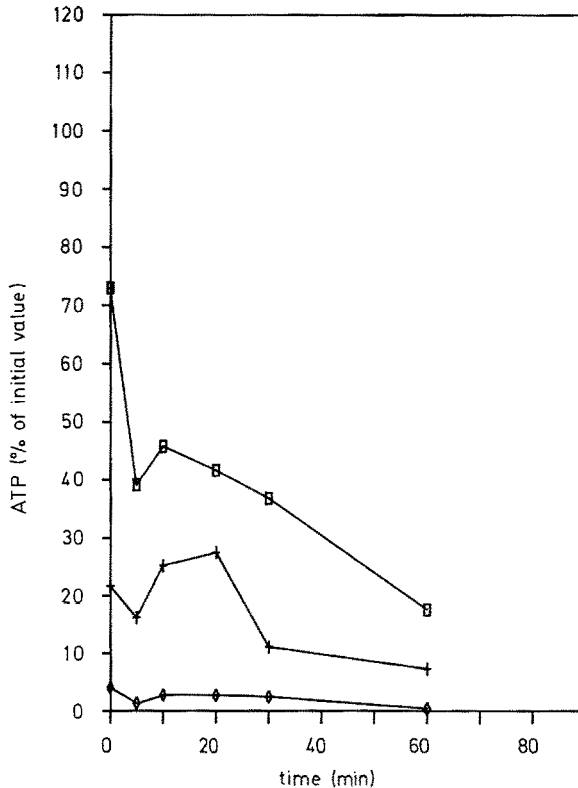


Fig. 4. The experimental conditions are as described in Fig. 3, with (—□—) representing the intracellular ATP concentration as a function of time after a 1 minute, (—△—) a 5 minutes and (—◇—) a 10 minutes exposure of infected cells to low pH in presence of 1 mM KCN. Syncytium formation was observed after the 1 minute exposure. The 5 minutes pH 6 treatment induced partial polykaryocyte formation, whereas a 10 minutes exposure to low pH prevented cell-cell fusion

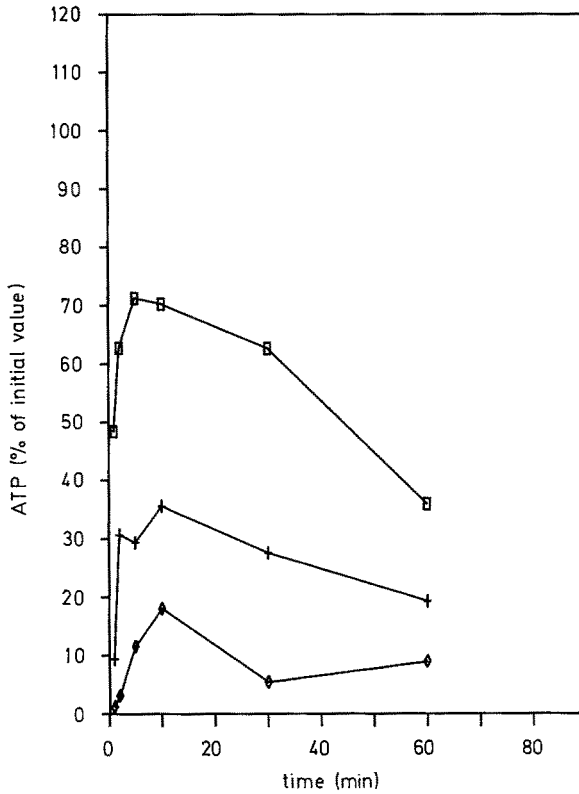


Fig. 5. The experiment was performed as described in Figs. 3 and 4. (—□—) ATP concentrations after a 5 minutes, (—+—) after a 30 minutes and (—◇—) after a 60 minutes exposure time to PBS/glucose, pH 6, containing 1 mM NaN_3 . As in Fig. 4, a short exposure allowed polykaryocyte formation, whereas with a 30 minutes exposure time only partial fusion was observed. The longest exposure time led to a failure of syncytium formation

After a 5 minutes exposure, syncytium formation was partially inhibited, whereas after 10 minutes at pH 6 it was totally blocked. When 1 mM NaN_3 was used as an inhibitor the time to deplete the cells was prolonged by a factor of up to 5 in the experiment shown in Fig. 5. All exposure times allowed a partial restoration of the cellular ATP concentration, but syncytium formation occurred only after the 5 minutes and partially after the 30 minutes exposure, whereas a longer pH 6 treatment in presence of NaN_3 (60 minutes) prevented polykaryocyte formation.

*Influence of 2-Deoxyglucose on Intracellular ATP and
SFV-induced Polykaryocyte Formation*

2-deoxyglucose is known to be a competitive inhibitor of glycolysis and induces a partial depletion of cellular ATP. Thus *Aedes* cells were infected with SFV and 16 hours later the growth medium was exchanged for PBS pH

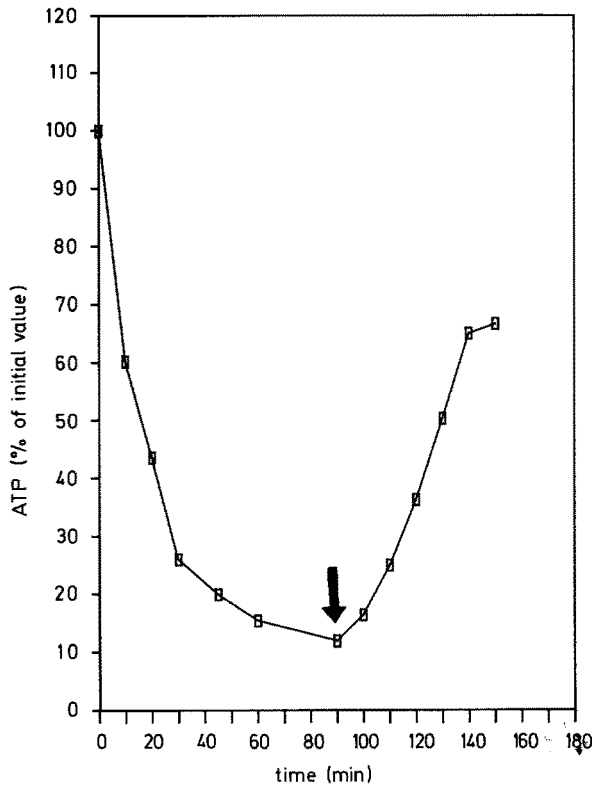


Fig. 6. The growth medium of SFV-infected cells was replaced at 16 hours p.i. by PBS pH 7.2 containing 50 mM 2-deoxyglucose. The intracellular ATP concentration was determined at regular intervals. After 90 minutes either the pH was lowered to 6 or the PBS/2-deoxyglucose was exchanged for MM medium. Lowering the pH to 6 resulted in a strongly hampered and delayed, partial fusion, whereas the addition of MM medium led to a restoration of the intracellular ATP level. 60 minutes after the addition of MM medium the pH was lowered to 6 resulting in polykaryocyte formation within 30 minutes

7.2 containing 50 mM 2-deoxyglucose. Cellular ATP concentration was measured over 90 minutes. As shown in Fig. 6, the cellular ATP level decreased to approximately 10 to 20 per cent of the initial value. Lowering the pH to 6 at this time resulted in a greatly delayed and hampered fusion, as judged by light microscopy. However, if the PBS containing 2-deoxyglucose was replaced by MM medium after 90 minutes, ATP recovered to almost 70 per cent of the initial level and a subsequent change to pH 6 after 1 hour led to a syncytium formation within 30 minutes.

Discussion

Syncytium formation of SFV-infected *Aedes albopictus* cells at mildly acidic pH can be inhibited by a wide variety of chemicals (11). Some of these drugs are known to deplete cells of ATP. In this report, we demonstrate that

depletion of cellular ATP in SFV-infected cells is accompanied by the failure of these cells to form polykaryocytes under conditions which normally do lead to cell-cell fusion. The experiments using inhibitors of oxidative phosphorylation clearly showed that blockage of *de novo* synthesis of ATP in the respiratory chain also prevents polykaryocyte formation triggered at low pH. The rapid depletion of cellular ATP of infected cells at pH 6 in presence of the inhibitors can be explained by the assumption that most ATP is used to counterbalance the intracellular pH to its normal level in presence of a large artificial pH gradient across the membrane (manuscript in preparation). When this pH gradient was abolished after a limited time of exposure to low pH in presence of the inhibitory drugs, the cellular ATP concentration recovered to a significant degree depending on the time of exposure to pH 6. This partial restoration of the intracellular ATP level in presence of inhibitors of oxidative phosphorylation can be explained by the fact that ATP cannot only be generated in the process of oxidative phosphorylation but also from other energy-rich compounds by transphosphorylations. It is noteworthy that these exposure times leading to different depletion levels varied with all inhibitors from experiment to experiment and were most probably dependent on the age of the culture. Furthermore, restoration of the intracellular ATP level was only transient in all experiments. The observed decrease after partial recovery of the ATP concentration can be explained by the need for ATP in the process of polykaryocyte formation per se and exhaustion of the energy reserves in the cell as mentioned above.

The experiments with 2-deoxyglucose clearly show that the ATP concentration can be restored, which then allowed the syncytium formation again. As a rule, the process of cell-cell fusion was never totally abolished, even at 10 to 20 per cent of the initial ATP level. This ATP concentration corresponds to the maximal depletion achievable by 2-deoxyglucose. The reason why the polykaryocyte formation was only greatly hampered and delayed but not completely inhibited can be explained by assuming that the residual amount of ATP synthesized by the oxidative phosphorylation, which itself is not blocked under these conditions, was just sufficient for cell-cell fusion to occur. The possibility that ATP depletion prevents polykaryocyte formation by blocking viral protein synthesis can be ruled out. KOBLET and collaborators (11) showed that addition of cycloheximide 16 hours after infection does not prevent syncytium formation during the next 8 hours.

In general, conditions which do not lead to an ATP depletion of more than approximately 80 per cent will allow SFV-induced polykaryocyte formation. Thus, the experiments strongly support the notion that SFV-induced syncytium formation is an ATP-consuming process. This finding is further supported by an earlier observation made by OKADA (13). It was reported that Sendai virus-induced polynuclear cell formation of Ehrlich's ascites tumor cells was inhibited by the addition of dinitrophenol.

Obviously, the results reported so far are not the reflection of the dynamic process of fusion, but of its static endproduct, the polykaryon. Thus, it could be argued that it is not the fusion event itself which requires ATP but the reorganization of the cellular membranes and the cytoplasm leading to a syncytium. However, we have shown that potassium cyanide inhibits polykaryon formation at a very early stage, namely at the level of membrane-membrane fusion (manuscript submitted). Briefly, SFV-infected cells were microinjected with the highly fluorescent, non permeable dye Lucifer yellow 1 minute after exposure to pH 6. Cell-cell fusion at this time could be observed by the spreading of the dye through microscopic connections into neighbouring cells. Concomitant addition of potassium cyanide with the change of the pH from 7 to 6 abolished this early process. Thus, these observations taken together lead to the conclusion that SFV-induced cell-cell fusion is a process which requires a specific expenditure of energy. Recent reports concerning other fusion phenomena also showed that these processes were energy dependent. A study by HERTEL and coworkers demonstrated the involvement of cellular ATP in receptor mediated endocytosis (7). DAVEY and collaborators showed that endocytotic fusion events require ATP (2). Furthermore, it is well known that fusion of secretory granules in chromaffine cells occurs only in presence of Mg-ATP (9).

Thus, comparing the striking parallelism between the different fusion phenomena, it is tempting to speculate that physiological and pathological fusions are ATP dependent. Additionally, our experiments lend further support to the notion that biological fusion processes are not only strictly physicochemical events—as could be deduced from reconstituted systems (e.g., liposome fusion)—but also include complex cellular events. However, the individual steps in membrane-membrane fusion requiring ATP and the final products of this metabolic process are as yet unknown.

Acknowledgements

This work was supported in part by the grant 3.384-0.82 from the Swiss National Science Foundation.

References

1. BESTERMAN JM, LOW RB (1983) Endocytosis: a review of mechanisms and plasma membrane dynamics. *Biochem J* 210: 1–13
2. DAVEY J, HURLEY SM, WARREN G (1985) Reconstitution of an endocytotic fusion event in a cell-free system. *Cell* 43: 643–652
3. FINKELDEY W (1931) Über Riesenzellbefunde in den Gaumenmandeln, zugleich ein Beitrag zur Histopathologie der Mandelveränderungen im Maserninkubationsstadium. *Virchows Arch Pathol Anat Physiol Klin Med* 281: 323–329
4. GOLDSTEIN JL, ANDERSON RG, BROWN MS (1979) Coated pits, coated vesicles and receptor-mediated endocytosis. *Nature* 279: 679–685

5. GUCCION JG, ENZINGER FM (1972) Malignant giant cell tumor of soft parts. *Cancer* 29: 1518–1529
6. HENLE G, DEINHARDT F, GIRARDIA A (1954) Cytolytic effects of mumps virus in tissue cultures of epithelial cells. *Proc Soc Exp Biol Med* 87: 386–393
7. HERTEL C, COULTER SJ, PERKINS JP (1986) The involvement of cellular ATP in receptor mediated internalization of epidermal growth factor and hormone-induced internalization of α -adrenergic receptors. *J Biol Chem* 261: 5974–5980
8. IGARASHI A (1978) Isolation of a Singh's *Aedes albopictus* cell clone sensitive to Dengue and Chikungunya viruses. *J Gen Virol* 40: 531–544
9. KNIGHT DE, BAKER PF (1982) Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J Membr Biol* 68: 107–140
10. KOBLET H, KEMPF C, KOHLER U, OMAR A (1985) Conformational changes at pH 6 on the cell surface of Semliki Forest virus-infected *Aedes albopictus* cells. *Virology* 143: 334–336
11. OMAR A, FLAVIANO A, KOHLER U, KOBLET H (1986) Fusion of Semliki Forest virus infected *Aedes albopictus* cells at low pH is a fusion from within. *Arch Virol* 89: 145–159
12. OKADA Y (1962) Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich's ascites tumor cells. I. Microscopic observation of giant polynuclear cell formation. *Exp Cell Res* 26: 98–107
13. OKADA Y (1962) Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich's ascites tumor cells. III. Relationship between cell condition and fusion reaction or cell degeneration reaction. *Exp Cell Res* 26: 119–128
14. OSHIRO LS, SCHIEBLE JH, LENETTE EH (1971) Electron microscopic studies of a coronavirus. *J Gen Virol* 12: 161–168
15. POSTE G, NICHOLSON GL (1978) Membrane fusion. In: *Cell surface reviews*, vol. 5. Elsevier/North Holland, Amsterdam
16. ROIZMAN B (1962) Polykaryocytosis. *Cold Spring Harbor Symp Quant Biol* 27: 327–342
17. STEINMAN RM, MELLMAN IS, MULLER WA, COHN ZA (1983) Endocytosis and recycling of plasma membrane. *J Cell Biol* 96: 1–27
18. WALTERS A (1983) Osteoclast-type giant cell tumor of the pancreas. *Acta Cytol* 27: 400–404
19. WHITE J, MATLIN K, HELENUS A (1981) Cell fusion by Semliki Forest, influenza, and vesicular stomatitis virus. *J Cell Biol* 89: 674–679

Authors' address: Dr. C. KEMPF, Institute for Hygiene and Medical Microbiology, University of Bern, Friedbühlstrasse 51, CH-3010 Bern, Switzerland.

Received November 4, 1986