ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 1978, p. 727-730
0066-4804/78/0014-0727$02.00/0

Vol. 14, No. 5
Printed in U.S.A.

Reversible Effects on Cellular Metabolism and Proliferation by Trisodium Phosphonoformate

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Received for publication 16 August 1978

The antiviral compound trisodium phosphonoformate (PFA), which inhibits herpesvirus multiplication by 50% at a concentration of 10 μM, did not show any effects on macromolecular synthesis and cell proliferation in HeLa and human lung cells at this concentration. At the high concentration of 2 mM, PFA reduced DNA synthesis to 50% after 1 h of treatment, whereas no effects could be seen on RNA and protein synthesis. Treatment for 24 h with 1 mM PFA inhibited both DNA synthesis and cell proliferation to 50%. The inhibition of DNA synthesis and cell proliferation at 10 mM PFA was rapidly reversed by removing the drug from the cells.

Trisodium phosphonoformate (PFA) (Fig. 1) has been shown to selectively inhibit herpesvirus-induced DNA polymerase (1a, 4) and influenza virus RNA polymerase (1a). It has an antiviral effect on herpesvirus types 1 and 2, pseudorabies virus, and infectious bovine rhinotracheitis virus in cell culture (1a). PFA is also therapeutically active against cutaneous herpesvirus infections in the guinea pig (1a). In this paper we report the effects of high concentrations of PFA on cellular metabolism and proliferation in HeLa and primary human lung cells and the reversal of these effects.

MATERIALS AND METHODS

Isotopes and chemicals. [*methyl*-^{3}H]thymidine (20.0 Ci/mmol), [5,6-^{3}H]uridine (47.9 Ci/mmol), and L-[4,5-^{3}H]leucine (60.0 Ci/mmol) and Biofluor scintillation solution were all purchased from New England Nuclear, Boston, Mass. All other chemicals were of analytical grade. PFA was synthesized according to Nylén (3) at the Research and Development Laboratories of Astra Läkemedel.

Cell cultures. HeLa cells were grown in minimal Eagle medium (MEM) supplemented with 7% newborn calf serum, 2% wt/vol HEPES (*N*-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) buffer (pH 7.2), penicillin (120 μg/ml), and streptomycin (100 μg/ml). Fetal human lung cells were purchased from Statens Bakteriologiska Laboratorium, Stockholm, Sweden, and grown in minimal Eagle medium supplemented with 10% fetal calf serum. Buffers and antibiotics were added as described above. The lung cells were used in passages 10 through 14, and Hela cells and human lung cells were maintained in 5% CO_2 at 37°C.

Incorporation studies. Confluent monolayers were trypsinized, and 2 ml of 1×10^5 cells per ml of suspension was transferred to each petri dish (Nunc, 3 cm in diameter). The growth medium was discarded and 2 ml of fresh medium containing serum was added immediately before the experiments. Half-confluent petri dishes were treated in duplicate with the substance for a short and a long period (1 and 24 h). Labeling was done by a 10-min pulse with 10 μCi of radioactive precursor for DNA, RNA, and protein synthesis. The incorporation was terminated by removal of medium, and each dish was washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS) and three times with 2 ml of ice-cold 5% trichloroacetic acid for 5 min each. The cells were then loosened and treated with 1 ml of 0.3 M NaOH per dish at 80°C for 15 min. Samples were counted in 10 ml of Biofluor, and the results were presented as incorporation into the trichloroacetic acid-insoluble product as compared to control cultures without PFA. For reversibility studies, cells inhibited with PFA were washed twice with 2 ml of PBS at 37°C, fresh medium with serum was added, and the incubation was continued. Labeling at indicated times was done as described above.

Cell proliferation. Twelve hours before the experiment, each dish was seeded with approximately 0.8×10^5 cells and incubated overnight to assure that the growth rate was normal. Incubation with PFA started at a cell number of approximately 1.6×10^5 cells per dish. At indicated times two dishes were washed with 2 ml of PBS each and mildly trypsinized for 5 min at 37°C. The trypsinized cells were diluted to 10 ml in particle-free buffer from Baker Diagnostics, Winchester, and counted in an electronic cell counter, model 134, from Analysinstrument AB, Stockholm, Sweden. When reversal was studied, the inhibited cells were washed twice with 2 ml of prewarmed PBS per dish, fresh medium with serum was added and incubation was continued up to 24 h.

RESULTS

Inhibition of macromolecular synthesis by PFA. Two inhibition periods were chosen to observe early and late effects on macromolecular synthesis. Incubation of human lung cells with 2 mM PFA for 1 h gave 50% inhibition of DNA synthesis, and no effects could be seen on RNA and protein synthesis (Fig. 2a). After 24 h of treatment with 1 mM PFA, DNA synthesis was inhibited 50%, and inhibition of RNA and protein synthesis could be detected at concentrations exceeding 2.5 mM (Fig. 2b). HeLa cells were affected in the same way as the lung cells (data not shown).

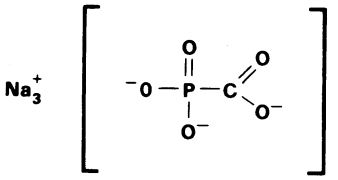

FIG. 1. *Chemical structure of PFA.*

Reversion of the inhibition of DNA synthesis by PFA. When HeLa cells (Fig. 3a) and human lung cells (Fig. 3b) were treated with 10 mM PFA for 1 h, the same inhibition level of DNA synthesis was obtained for both cell types. Immediately after removal of the drug, DNA synthesis started and reached the normal level within 5 h.

Effect on cell proliferation by PFA. As was the case for inhibition of macromolecular synthesis, very high concentrations of PFA had to be used to decrease the growth rate of human lung cells (Fig. 4). Cell proliferation was not affected at a concentration of 0.1 mM PFA, whereas the growth rate was reduced to about 50% of the control at 1 mM PFA. At 10 mM, the cellular growth rate was almost completely inhibited, and after 36 h the cells tended to loosen

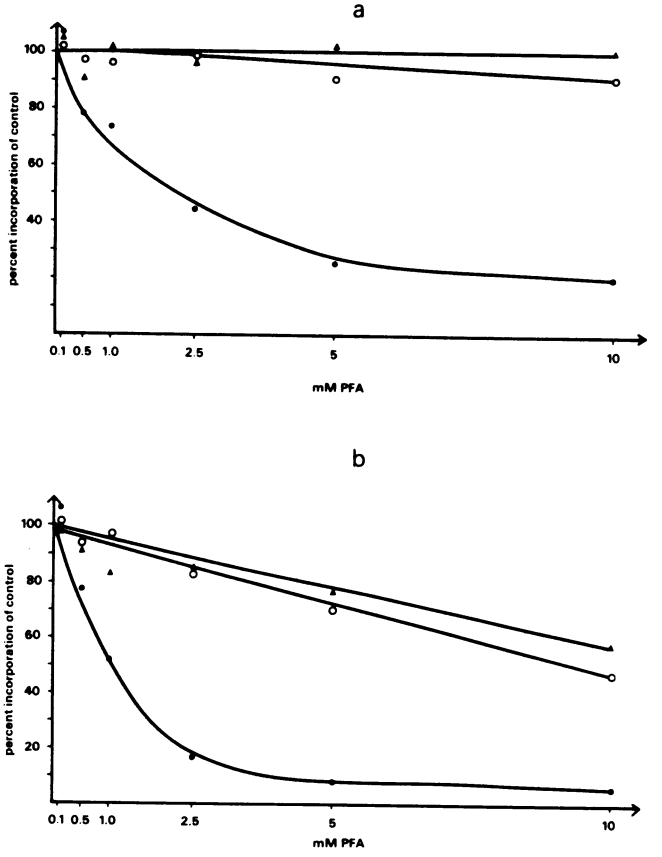

FIG. 2. *Effect of PFA on macromolecular synthesis in human lung cells. (a) Incubation for 1 h with PFA and pulse-labeling for 10 min. (b) Incubation for 24 h with PFA and pulse-labeling for 10 min. Symbols: ●, [^{3}H]thymidine; ▲, [^{3}H]uridine; ○, [^{3}H]leucine.*

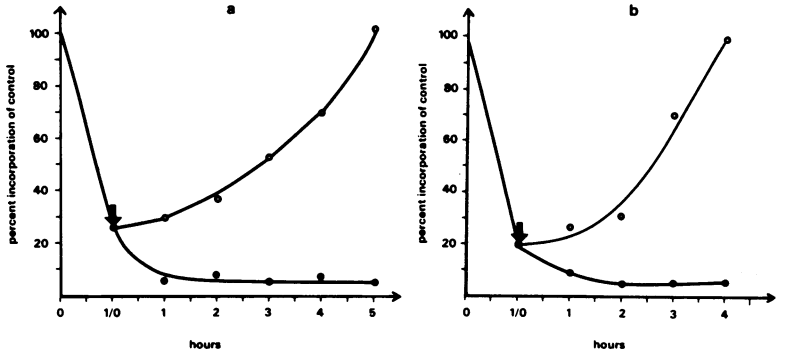

FIG. 3. *Reversion of PFA inhibition of DNA synthesis. HeLa cells (a) and human lung cells (b) were inhibited with PFA, washed, and incubated without the drug. The cells were labeled with [^{3}H]thymidine for 10 min at indicated times. (a) HeLa cells. Symbols: ○, 10 mM PFA removed after 1 h (arrow); ●, 10 mM PFA present. (b) Human lung cells. Symbols: ○, 10 mM PFA removed after 1 h (arrow); ●, 10 mM PFA present.*

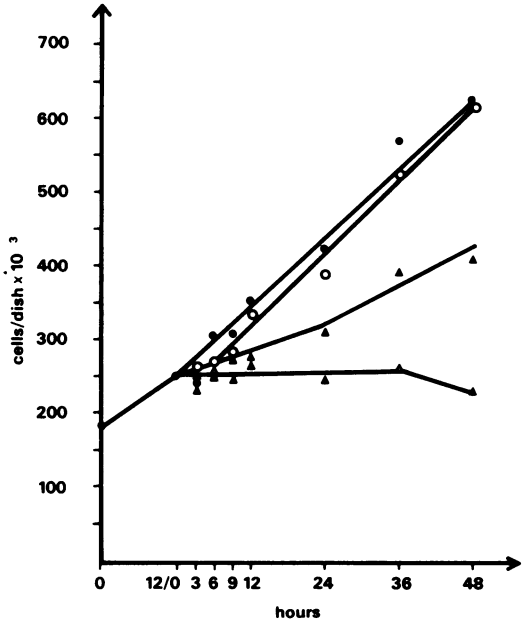

FIG. 4. *Effect on cell proliferation by PFA. Monolayer cultures with human lung cells were incubated with PFA. At indicated times plates were counted in a cell counter. Symbols: ●, Control; ○, 0.1 mM PFA; ▲, 1 mM PFA; △, 10 mM PFA.*

from the surface. HeLa cells were affected in the same way as human lung cells (data not shown).

Reversal of the inhibition on cell proliferation by PFA. Cells inhibited with 10 mM PFA for 24 h had a completely blocked cell division. After the drug was removed, cell division started, and after 9 h, the growth rate was equal to that of the control for both cell types (Fig. 5a and b).

DISCUSSION

In the search for new antiviral agents it is important to determine any effects on cellular functions. A concentration of 10 μM PFA has been shown to give 50% plaque reduction of herpesvirus in cell cultures (1a). At this concentration no effects on macromolecular synthesis or cell proliferation could be detected in uninfected cells. To detect any effects of PFA on cell cultures very high concentrations, even up to 10 mM, had to be used. Our results show that only DNA synthesis was affected after 1 h of treatment with PFA (Fig. 2a). This is in agreement with Sabourin et al. (5), who reported that PFA inhibits DNA polymerase α of human cells. After 24 h of treatment, DNA, as well as RNA, and protein synthesis were inhibited at high concentrations of PFA (Fig. 2b). A possible conclusion from these results is that high concentrations of PFA primarily affect DNA synthesis and that RNA and protein syntheses are inhibited later as a consequence of the reduced DNA synthesis Another explanation is that, after treatment for 24 h with high concentrations of PFA, the cell number is reduced, and since the incorporation is compared to that in the untreated controls, this could also explain the greater inhibition after 24 h.

DNA synthesis inhibited with 10 mM PFA for 1 h was completely reversed 5 h after removal of the drug from the cells (Fig. 3a and b). The same reversal time was also observed after 24 h of inhibition with the same concentration (data not shown).

To extend the results from PFA's effect on macromolecular synthesis, cell proliferation was studied and was found to be affected to the same extent as DNA synthesis. Cells incubated for 24

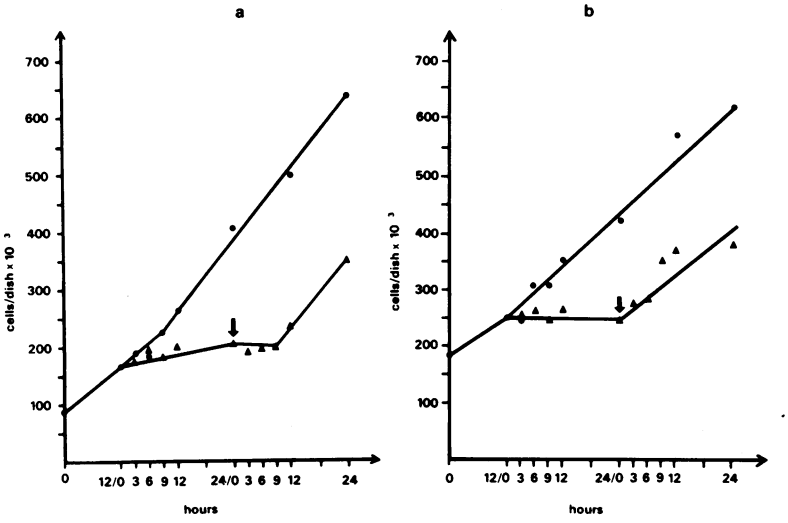

FIG. 5. *Reversion of the inhibition on cell proliferation by PFA. Monolayer cultures with HeLa cells (a) and human lung cells (b) were incubated with PFA, washed, and incubated without the drug. Symbols: ●, Control; △, 10 mM PFA removed after 24 h (arrow).*

h with 10 mM PFA completely blocked cell division. However, this effect could be rapidly reversed by drug removal (Fig. 5a and b). These results imply that PFA was not accumulated within the cell and that no irreversible damage had occurred.

A comparison between PFA and phosphonoacetic acid, idoxuridin, vidarabine, and ribavirin (1a) has shown that PFA has a low cytotoxicity and a favorable ratio between cell toxicity and inhibition of herpesvirus multiplication. We could not observe any difference in effect of PFA on cell metabolism and proliferation between HeLa cells and human lung cells.

Our results show that PFA has no cytotoxic effects at concentrations giving an antiviral effect and that inhibition of macromolecular synthesis and cell proliferation at high concentrations of PFA (10 mM) can be rapidly and completely reversed by removing the drug from the cells.

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