Antiviral Effect of Prostaglandins of the A Series: Inhibition of Vaccinia Virus Replication in Cultured Cells

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SUMMARY

Prostaglandins of the A series potently inhibited the production of vaccinia virus in mouse L fibroblasts. With the highest non-toxic dose of PGA₁, 4 µg/ml, the replication of the virus was inhibited by 95.3%. The antiviral activity was dose-dependent and specific for the A series. At the dose used, PGA₁ was not toxic to uninfected cells and did not alter cell metabolism as measured by DNA, RNA and protein synthesis. PGA₁ did not influence the adsorption of the virus by the host cells and the antiviral activity was not dependent on the presence of PGA₁ during the early stages of infection. PGA₁ treatment delayed and partially inhibited virus DNA synthesis and, while it did not produce any change in the pattern of protein synthesis in uninfected cells, it altered both the rate and the pattern of virus protein synthesis. We conclude that PGA₁ selectively inhibits one or more steps involved in the replication of vaccinia virus in mouse L fibroblasts.

INTRODUCTION

The possibility that some prostaglandins (PGs) affect virus replication in cultured cells has recently been investigated. Harbour et al. (1978) have shown that prostaglandins of the E and F series increase the size of herpes simplex virus (HSV) plaques in Vero cells; an increase in the yield of the virus in the presence of PGE₂ was noted only at low multiplicities of infection (m.o.i.), while there was no effect reported on the production of some RNA viruses (measles virus and coxsackie virus B₃). In contrast, inhibition by PGE of the production of parainfluenza 3 virus in WISH cells was reported by Luczak et al. (1975); these investigators suggested that this effect was related to an alteration in the rate of cell growth and was mediated by changes in cellular cAMP synthesis. In previous studies, we have demonstrated that prostaglandins of the A series (PGA) are potent inhibitors of Sendai virus replication in African green monkey kidney (AGMK) cells in culture (Santoro et al., 1980, 1981). This antiviral activity was specific for prostaglandins of the A series and was dose-dependent; furthermore, the highest effective dose, 4 µg/ml, was not toxic to the cells, nor did it alter host cell metabolism or infectivity of the virus. Finally, PGA₁ prevented the establishment of persistent infection in this system.

In order to determine whether PGA antiviral action is limited to one particular system or has a broader spectrum of activity, we have investigated its effect on different viruses. In the present study, we report that PGA₁ potently inhibited virus replication in vaccinia-infected mouse L fibroblasts and examined the possible sites of action of this prostaglandin.

METHODS

Cell culture. Mouse L fibroblasts were grown in monolayer in T-75 Falcon flasks or in 24-well Linbro plates, in Eagle's minimal essential medium (MEM) supplemented with 10% newborn calf serum (NCS; Gibco), at 37 °C in a 5% CO₂, 95% air atmosphere. These cells normally synthesize both PGE₂ and PGA₂, as measured by radioimmunoassay after organic solvent extraction and silicic acid chromatography (Jaffe et al., 1973).
Virus. Vaccinia virus, strain WR, was grown in HeLa cells and purified according to the technique of Joklik (1962). Cells were infected at 500 particles (10 p.f.u.) per cell, for 1 h at 37 °C, after which time the virus inocula were removed and media containing 2% NCS and PGA, or ethanol diluent were added.

Prostaglandins. Prostaglandins were obtained from the Upjohn Co., Kalamazoo, Mi., U.S.A. The synthetic PGE1 and PGA, analogues (16,16-dimethyl esters) were a generous gift of Dr J. Pike, the Upjohn Co. The PGs were stored as 100% ethanolic stock solutions (10 mg/ml) at -20 °C and were diluted to the appropriate concentrations at the time of their use. Control media contained the same concentration of ethanol diluent (0-04%) which was shown not to affect either cell or virus metabolism. As determined by radioimmunoassay, PGs are stable in tissue culture media for at least 24 h.

Virus titration. Samples for virus titration were collected 24 h post-infection by detaching the cell monolayers with a rubber policeman. After sonication, virus suspensions were serially diluted and at least three dilutions (10^-4 to 10^-6) were used for plaque determination. Duplicate dishes were used for each dilution. Vero cell monolayers were infected with diluted virus for 1 h at 37 °C and, after the inocula were removed, Dulbecco's modified MEM containing 3% NCS and 0-9% agar (Bacto-Agar; Sigma) was added. Plaques were scored after 7 days.

DNA, RNA and protein synthesis. The kinetics of DNA, RNA and protein synthesis in uninfected as well as infected cells were monitored in 24-well Linbro plates, in confluent cells. At the times indicated in the text, cells were pulse-labelled (10 min) with 10 μCi/ml [3H]thymidine, [3H]uridine or [35S]methionine for DNA, RNA or protein synthesis, respectively, and the radioactivity incorporated into acid-soluble and -insoluble material was determined. Cells were washed three times with phosphate-buffered saline (PBS) and 0-5 ml 5% trichloroacetic acid (TCA) was added to each culture. After 1 h, 0.2 ml volumes of acid-soluble material were counted in Aquasol (New England Nuclear). Acid-insoluble radioactivity was measured after washing the TCA precipitates with ethanol three times, drying under an infrared lamp and dissolving the samples in 0.5 ml of a solution containing 0-1 m-NaOH, 0.5% SDS. Samples of 0.2 ml were counted in Aquasol.

Staining of virus 'factories'. The sequential appearance, size and location of the virus 'factories' in PG-treated and untreated infected cells were observed by microscopy using a fluorescent DNA-binding compound, Hoechst 33258, as previously described (Esteban, 1977).

Synthesis of virus DNA. Monolayers of cells grown in 100 mm dishes were infected with 1000 particles/cell of purified vaccinia virus in 1 ml MEM. After 1 h of virus adsorption at 37 °C, the inocula were removed and replaced with 10 ml MEM containing 2% NCS with or without prostaglandins. For the preparation of labelled virus, cells were labelled with 2 μCi/ml (20 μCi/plate) of [3H]thymidine 2 h post-infection. At 24 h post-infection, cells were washed three times in PBS, scraped and resuspended in 1 ml PBS containing 1% Nonidet P40. At 10 min at 4 °C, samples were centrifuged (2000 rev/min for 10 min). The cytoplasmic extracts were sonicated and then loaded on linear 20 to 45% (w/v) sucrose gradients in 1 mM-NaHPO4. Samples were centrifuged at 15000 rev/min for 15 min at 4 °C (Spinco L250, SW41 rotor) and fractions were collected. Carrier bovine serum albumin (BSA) was added to each fraction and samples were precipitated with 10% TCA for 1 h at 0 °C. The precipitates were collected on Whatman GF/C filters, washed three times with 5% TCA, dried and counted in a toluene-based scintillation mixture.

For the kinetics of virus DNA synthesis the same technique was used, but samples were pulse-labelled (10 min pulses) every hour for a period of 6 h after infection. Before sonication, aliquots of the cytoplasmic extracts (0-1 ml) were TCA-precipitated and the radioactivity was counted after filtration on Whatman GF/C filters.

Polyacrylamide gel electrophoresis (PAGE). Proteins were labelled with [35S]methionine as previously described (Metz & Esteban, 1972). Samples were analysed by SDS-PAGE in a vertical slab gel apparatus, using the buffer system described by Laemmli (1970). The 3% stacking gel and 7 to 14% resolving gradient gels were run at 120 V. The gels were washed, fixed in 10% acetic acid, 10% TCA and 30% methanol, dried under vacuum and autoradiographed using DuPont Cronex Films.

RESULTS

Effect of prostaglandins on vaccinia virus production

We tested the effect of a number of prostaglandins and PG-related compounds on vaccinia virus production. Prostaglandins of the E, F, B, D series and thromboxane B2 were ineffective (data not shown), but PGs of the A series (PGA1 and the 16,16-dimethyl PGA2 methyl ester analogue) were potent inhibitors of virus production. The effect of PGA1 on virus replication is shown in Fig. 1. Fig. 1(a) shows the titration of virus p.f.u. by plaque assay on monolayers of Vero cells, after treatment with PGA1 (4 μg/ml), 24 h post-infection. This dose was the most effective non-toxic dose, caused 95.3% inhibition of virus production and was used in all subsequent experiments. No differences in the size of the plaques were noted. Fig. 1(b) shows the sedimentation profile of [3H]thymidine-labelled virus particles after analysis of the
Fig. 1. Inhibition of vaccinia virus production by PGA₁. (a) Titration of virus yields by plaque assay on Vero cells after 24 h treatment with 4 µg/ml PGA₁. Samples were diluted to 10⁻⁴, 10⁻⁵ and 10⁻⁶ and four dishes were tested for each group, at each dilution. (b) Sedimentation profiles of [³H]thymidine-labelled material after loading the cytoplasmic extracts of vaccinia-infected cells on continuous 20 to 45% sucrose gradients. Sedimentation was from right to left. The virus peak was between fractions 11 and 15. Samples were treated with the indicated doses of PGA₁ for 24 h.

cytoplasmic extracts from infected cells on continuous 20 to 45% sucrose gradients (see Methods). While there was no effect with doses as low as 0.1 µg/ml, the size of the virus peak (between fractions 11 and 15) was substantially reduced following treatment with 2 µg/ml PGA₁ and this peak was completely abolished at the 4 µg/ml dose. Comparable data were obtained when virus yields were titrated by plaque assay in these samples [control, 17.3 ± 2.4; 0.1 µg PGA₁, 13.6 ± 1.5; 2 µg PGA₁, 3.1 ± 0.4 and 4 µg PGA₁, 0.82 ± 0.2 p.f.u. × 10⁶/ml]. That the range of doses required for the antiviral action was supraphysiological suggests the PGA₁ action to have been pharmacological.

Effect of PGA₁ on macromolecular synthesis in uninfected and vaccinia virus-infected L cells

The addition of 4 µg/ml PGA₁ to the culture medium of uninfected L cells did not produce any toxic effect, as determined by either microscopic examination or the vital dye exclusion technique. Treatment for periods up to 24 h with PGA₁ at this dose did not alter either the
uptake of precursors or the synthesis of DNA, RNA or protein in these cells, as measured by the incorporation of \(^{3}H\)thymidine, \(^{3}H\)uridine or \(^{35}S\)methionine respectively (data not shown).

In WR-strain-infected L cells grown in monolayer, the cytopathic effect became evident as early as 90 min after infection. The appearance of the cytopathic effect was generally delayed for a period of 1 to 3 h after administration of PGA\(_1\). After 24 h, however, there were no differences in the cytopathic effects or in the rate of cell death in control and PGA-treated cells.

At early times after infection (1 to 5 h), no differences were found in the uptake of thymidine in control and PG-treated cells. However, PGA\(_1\) significantly inhibited DNA synthesis at 2 and 3 h after infection and enhanced both the uptake of \(^{3}H\)uridine and the rate of RNA synthesis in WR-infected cells. PGA\(_1\) treatment also produced an increase in the amount of \(^{35}S\)methionine (10 \(\mu\)Ci/\(10^6\) cells) in TCA-soluble material and a decrease 3 h post-infection in its incorporation into proteins.

PGA\(_1\) did not alter virus adsorption to the cells. Virus adsorption was measured by infecting L monolayers with \(^{3}H\)thymidine-labelled virus DNA (1 \(\times\) \(10^3\) particles/cell, sp. act. \(2.5 \times 10^4\) ct/min/\(\mu\)g DNA) in the presence of 4 \(\mu\)g/ml PGA\(_1\) for 1 h. After repeated washings with cold PBS, cells were treated with 5\% TCA, washed three times with absolute ethanol and dissolved in 0.4 ml of 0.5\% SDS in 0.1 \(M\)-NaOH. In quadruplicate samples, an average of 25550 \(\pm\) 2231 (\(^{3}H\)) ct/min/\(10^6\) cells were registered in control samples and 30676 \(\pm\) 1632 in PGA\(_1\)-treated cells.

Because PGA\(_1\) did not alter virus adsorption by the cells, and PG treatment as late as 90 min after infection was effective in inhibiting virus production, an action of PGA\(_1\) on an early event of virus replication [i.e. the entry, uncoating, and early RNA synthesis, which peaks at 30 to 60 min (Metz & Esteban, 1972)], was possibly not required for the antiviral effect. To determine at what stage PGA\(_1\) was inhibiting virus multiplication, we examined virus DNA and protein synthesis in infected cells. PGA\(_1\) treatment produced both a delay and a decrease (57\%) at 2 h post-infection in virus DNA synthesis, as measured at 1 h intervals by thymidine incorporation into cytoplasmic extracts. Four h after infection, 60\% inhibition of virus "factories" production was also noted. Since PGA only temporarily and partially inhibited vaccinia DNA synthesis, this inhibition could only have been partly responsible for the suppression of virus production. We were unable to detect any difference in virus RNA synthesis using the techniques described above.

The pattern of host and virus protein synthesis was analyzed after labelling the cells with \(^{35}S\)methionine by separation on SDS–polyacrylamide gels and autoradiography (see Methods). PGA\(_1\) did not alter the pattern of protein synthesis in uninfected L cells; however, numerous differences in the pattern of virus proteins of WR-infected control and PGA\(_1\)-treated cells were detected. Fig. 2 shows the polyacrylamide gel analysis of the polypeptides of uninfected (lane 1, control; lane 2, PGA) and vaccinia-infected cells, labelled at different times after infection. A number of early virus proteins (lane 3, virus control) were not synthesized by 1.5 h in the PGA\(_1\)-treated cells (lane 4). In these cells, some of these early proteins were synthesized later, at 3 h (lane 5, control; lane 6, PGA), while others were never synthesized. Some of the late proteins (5 h; lane 7, control; lane 8, PGA) were synthesized at the same rate in control and PG-treated cells, while some were not synthesized or were synthesized to a much reduced extent in PG-treated cells. \(^{35}S\)-labelled virion polypeptides are shown in lane 9 of Fig. 2 with an indication of their molecular weights in order to facilitate comparison with virus structural proteins in infected cells.

**DISCUSSION**

Prostaglandins are a group of naturally occurring cyclic 20-carbon fatty acids. They are characterized as mono-, bi- and tri-unsaturated compounds by the number of C–C double bonds in the aliphatic side chains, and are designated by differences in the structure of the cyclopentane ring. Prostaglandins are synthesized in most cell types and they have been shown to be involved in the control of a spectrum of physiological phenomena, including regulation of cell growth and differentiation (Jaffe & Santoro, 1977), immune functions (Goodwin & Webb, 1980), interferon action (see below) and others.

The possibility of a relationship between prostaglandins and virus-mediated functions was
PGA₁ inhibits vaccinia virus replication

Fig. 2. Polyacrylamide gel analysis of polypeptides synthesized in cells infected with vaccinia and treated with PGA₁. PG treatment was started after the 1 h adsorption period. Cells were labelled at different times as described in the text (1 h pulse labelling with 10 μCi/ml [¹⁵S]methionine). 'U' denotes uninfected cells (lane 1, control; lane 2, PGA-treated); lanes 3, 5 and 7 represent samples from vaccinia virus-infected cells at 1-5, 3 and 5 h post-infection respectively; lanes 4, 6 and 8 represent samples from vaccinia virus-infected cells treated with PGA₁, at 1-5, 3 and 5 h post-infection, respectively. Lane 9 represents ³⁵S-labelled virion polypeptides prepared from infected L cells, after 24 h of labelling and purified by the method of Joklik (1962). Molecular weights refer to the following markers: 14300 lysozyme; 18400 β-lactoglobulin; 24000 trypsinogen; 34700 pepsin; 45000 ovalbumin; 66000 bovine plasma albumin.

First suggested by the studies of Ritzi & Stylos (1976) and Hammarström (1977) who reported that transformation of Balb/c-3T3 fibroblasts by simian virus 40 and polyoma virus, respectively, substantially increased the rate of PG biosynthesis in these cells. Recent observations have established a relationship between prostaglandins and interferon. Yaron et al. (1977) demonstrated that inducers of interferon stimulated the synthesis of PGs, and Stringfellow (1978) reported that the addition of PGE₂, PGA₂ and PGF₂α restored the interferon response in hyporeactive animals. Moreover, interferon induction correlated closely with the amount of increased cellular PG production in seven distinct cell lines, infected with six viruses (Fitzpatrick & Stringfellow, 1980). Finally, two inhibitors of prostaglandin synthesis, indomethacin and aspirin, were shown to prevent the antiviral action of interferon in mouse L cells infected with vesicular stomatitis virus (Pottathil et al., 1980).

We have previously reported that prostaglandins of the A series are potent inhibitors of the production of Sendai virus in AGMK cells. In the present study we have demonstrated that PGAs also potently inhibit the replication of vaccinia virus in mouse L fibroblasts. These results suggest that the antiviral action of the PGAs may have a broad spectrum of action and be effective on several cell types.
The inhibition of vaccinia virus production was both dose-dependent and specific to prostaglandins of the A series. We have shown that PGA₁ did not influence the adsorption of virus by the host cells and its presence was not necessary in an early stage of infection for the antiviral action to occur. At the dose of PGA₁ used, this compound was not toxic to the uninfected cells and did not alter cell metabolism as measured by DNA, RNA and protein synthesis. In contrast, in infected cells, PGA₁ treatment partially prevented the vaccinia-induced 'shut-off' of RNA synthesis while it produced a decrease in both DNA and protein synthesis starting 2 h after infection. PGA₁ also caused a delay and partial inhibition of virus DNA synthesis and induced a radical change in both the rate of synthesis and in the pattern of virus proteins. While some of the virus proteins were not synthesized or appeared later, other virus proteins were produced at the same rate as in untreated, virus-infected cells, suggesting that these changes were not due to a non-specific toxic effect but rather to a specific block on protein synthesis.

The mechanism of the antiviral action of PGA on vaccinia virus-infected cells may be mediated at the level of transcription and/or translation of virus mRNAs. The understanding of the steps which lead to a selective inhibition of vaccinia virus proteins by PGAs may provide some insights into the mode of action of prostaglandins in animal cells.

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REFERENCES


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