Selective Inhibition of Viral Gene Expression as the Mechanism of the Antiviral Action of PGA₁ in Vaccinia Virus-infected Cells

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SUMMARY

We have previously shown that prostaglandins of the A series are potent inhibitors of the replication of several animal viruses in cultured cells. In this report we have studied the mechanism of the antiviral action of PGA₁ in vaccinia virus-infected mouse L cells, where there is an alteration in both the rate and extent of the synthesis of some virus proteins. When cytoplasmic RNAs from PGA-treated, vaccinia virus-infected cells were translated in cell-free systems, similar selective inhibition of the synthesis of some viral polypeptides was observed. The lack of translation of some viral RNAs was not due to an impairment of the methylation process nor to a difference in ionic requirements. PGA₁, even at doses as high as 10 μg/ml, did not exert any direct inhibitory action on transcription in vitro as measured in two cell-free systems, and had no effect on primary transcription—translation of vaccinia virus RNAs when assayed in coupled cell-free systems. Southern blot hybridization analysis of cytoplasmic RNAs to EcoRI restriction fragments of vaccinia DNA showed that PGA₁ was able to induce major changes in the pattern of RNA transcripts during the course of viral infection. We propose that changes in the transcription programme of vaccinia virus RNAs could be due either to an alteration of specific viral proteins that regulate transcription by direct binding of PGA₁, or to the synthesis and/or activation of a host product that mediates the antiviral action.

INTRODUCTION

Prostaglandins (PGs) are a group of naturally occurring cyclic 20-carbon fatty acids that are synthesized by most types of cells and 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 function (Goodwin & Webb, 1980), interferon action (Stringfellow, 1978; Fitzpatrick & Stringfellow, 1980; Pottathil et al., 1980) and others. The possibility that some prostaglandins 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, while no effect was found on the production of some RNA viruses (measles virus and coxsackie virus B₁). In contrast, inhibition of the production of parainfluenza 3 virus by PGE in Wish cells was reported by Luczak et al. (1975).

In previous studies, we have demonstrated that pharmacological doses of prostaglandins of the A series potently inhibit replication of Sendai virus in African green monkey kidney cells (Santoro et al., 1980, 1981) and of vaccinia virus in mouse L fibroblasts (Santoro et al., 1982a). The antiviral effect was specific for prostaglandins of the A series (both PGA₁ and A₂) and dose-dependent. The most effective non-toxic dose, 4 μg/ml, produced 95-3% inhibition of vaccinia virus replication, as measured by plaque titration on Vero cells. In uninfected L cells,
treatment with this dose of PGA, for periods up to 24 h did not cause significant changes in the uptake of precursors and in the synthesis of DNA, RNA and proteins (Santoro et al., 1982a). In vaccinia virus-infected L cells, the same dose of PGA, did not alter the adsorption, penetration, or uncoating of the virus while it delayed and partially inhibited viral DNA synthesis. A further increase in the virus-induced 'shut-off' of protein synthesis was also shown (Santoro et al., 1982a). While in uninfected cells there was no change in the pattern of the proteins synthesized, in vaccinia virus-infected cells, PGA, produced a general change in the rate and extent of production of the viral polypeptides. Some viral proteins were not synthesized or appeared later, while other viral proteins were produced at the same rate as in control cells, suggesting that these changes were not due to a non-specific toxic effect but rather to a specific block in protein synthesis. This effect could be due to an inhibition of the transcription and/or the translation of viral RNAs. In order to determine at what level prostaglandins exert their inhibitory action, we have characterized, by translation in a cell-free system and by DNA–RNA hybridization analysis, the nature of viral RNAs synthesized in prostaglandin-treated cells. The data showed that PGA, was able to induce major changes in the pattern of RNA transcripts during the course of viral infection.

**METHODS**

**Cell culture.** Mouse L fibroblasts were grown in monolayer in Eagle's minimal essential medium (MEM) supplemented with 10% newborn calf serum (NCS, Gibco), at 37°C in a 5% CO₂ atmosphere.

**Prostaglandins.** PGA, (Sigma) was stored as a 100% ethanolic stock solution (10 mg/ml) at -20°C and was diluted to the appropriate concentration of ethanol diluent (0.04%), which was shown not to affect cell metabolism. As determined by radioimmunoassay, PGA, is stable in tissue culture medium for at least 24 h.

**Virus.** Vaccinia virus, strain WR, was grown in HeLa cells and purified according to the technique of Joklik (1962). Confluent monolayers of cells were infected at a m.o.i. of 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.

**RNA extraction.** RNA was isolated from vaccinia virus-infected or control L cells usually 4.5 h after infection (a time at which a complete pattern of synthesis of virus RNA and proteins was obtained), using a modification of the technique of Cabrera et al. (1978). Cytoplasmic extracts were prepared by Dounce homogenization in 20 mM-Tris–HCl pH 7.5, 3 mM-CaCl₂, 3 mM-MgCl₂, 0.5% Nonidet P40, 10 mM-EDTA, and nuclei were removed by centrifugation (Esteban & Metz, 1973). After addition of 0.4 M-NaCl and 0.2% SDS, supernatants were extracted as previously described (Cabrera et al., 1978); nucleic acids were precipitated by the addition of 2 vol. ethanol for 12 h at -20°C and resuspended in 200 μl of 10 mM-Tris–HCl pH 7–6, 10 mM-MgCl₂. The RNA concentration was determined spectrophotometrically assuming 1 A₂₆₀ unit is equivalent to 45 μg RNA/ml. Generally, about 100 μg of RNA was recovered from 1 × 10⁶ cells. The RNA was essentially protein-free as determined by the A₂₆₀/A₂₈₀ ratio.

**Cell-free protein synthesis.** The message-dependent rabbit reticulocyte lysate used for these experiments has been previously described (Cooper & Moss, 1979). Concentrations of cytoplasmic RNA ranging from 200 to 1000 μg/ml were used. [³⁵S]Methionine (Amersham) was added at a concentration of 10 μCi/25 μl sample. Samples were incubated at 30°C for 60 min and radioactive incorporation was measured by hot trichloroacetic acid (TCA) precipitation (Cooper & Moss, 1978).

**Polyacrylamide gel electrophoresis (PAGE).** Proteins labelled with [³⁵S]methionine were analysed by SDS–PAGE in a vertical slab gel apparatus, using the buffer system described by Laemmli (1970). The 3% stacking gel and 10 to 15% 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.

**Hybridization of RNA to restriction fragments of vaccinia DNA.** Purification of viral DNA, cleavage with the restriction endonuclease EcoRI, and analysis of the resulting fragments by electrophoresis in agarose gels have been previously described (McCarron et al., 1978). DNA fragments separated in agarose gels were denatured in situ and transferred to nitrocellulose filters by a modification (McAllister & Barrett, 1977) of the method of Southern (1975). Each filter strip received 1.5 to 1.7 μg of viral DNA. Viral RNAs were labelled for 4 h, starting 30 min after infection, with Na[³²P]PO₄ (Amersham) (1 mCi/2 × 10⁶ cells) in the presence or absence of PGA, (4 μg/10⁶ cells) and cycloheximide (100 μg/ml) (Flow Laboratories). After extraction, as described in the previous section, RNAs were resuspended in buffer (10 mM-Tris–HCl pH 7–6, 10 mM-MgCl₂), treated with 50 μg/ml DNase (Sigma) for 15 min at 37°C, re-extracted and precipitated with ethanol (Cabrera et al., 1978). Identical amounts of ³²P-labelled RNA were added to nitrocellulose strips, with the exception that hybridization was carried out in the
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presence of 5 × SSC (1 × SSC = 0.15 M-NaCl, 0.015 M-sodium citrate), 0-1 % SDS, 10 mm-sodium phosphate and 50 % formamide. After 24 h at 42 °C, the filter strips were removed, rinsed repeatedly, and then incubated in 2 × SSC solution containing 20 μg/ml pancreatic RNase (Sigma). Filter strips were autoradiographed using DuPont Cronex films.

Transcription in vitro by the vaccinia virion-associated RNA polymerase. Purified virions were collected by centrifugation and 2 × 10¹⁵ virus particles were resuspended at 0 °C in 50 mm-Tris-HCl buffer pH 8.6, 6 mm-MgCl₂, 7 mm-2-mercaptoethanol, 0.05 % Nonidet P40, 3 mm-ATP, 1 mm-GTP and -CTP, 60 μM-[³H]UTP (7.6 Ci/mmol) in a final volume of 0.4 ml (Kates & Beeson, 1970). The reaction mixtures were sonicated for 10 s (50 W, Biosonic ultrasonicator) and incubated at 37 °C with continuous shaking in the presence of PGA₁ or ethanol diluent. At appropriate intervals, aliquots were removed and the radioactive incorporation into hot TCA-precipitable material was determined (Cooper & Moss, 1978).

Transcription of vaccinia DNA with Escherichia coli RNA polymerase. The reaction mixtures (total volume of 100 μl) contained 80 μg/ml vaccinia virus DNA, 80 units/ml E. coli RNA polymerase (New England Nuclear) in 40 mm-Tris-HCl buffer pH 7.9, 10 mm-MgCl₂, 0-1 mm-EDTA, 0.1 mM-DTT (dithiothreitol), 150 mm-KCl, 0-2 mm-ATP, -GTP, -CTP and 60 μM-[³H]UTP. The reaction mixtures were incubated at 37 °C in the presence of PGA₁ or ethanol diluent and, at various times, aliquots were removed and processed for counting of TCA-precipitable radioactive material (Cooper & Moss, 1978).

Coupled transcription-translation cell-free system. Preparation of vaccinia virus cores and optimal conditions for the coupled cell-free system with rabbit reticulocytes were previously described (Cooper & Moss, 1978). Prostaglandin or ethanol diluent were added at appropriate concentrations and incubation was carried out at 30 °C for 2 h. Samples were then dissolved and processed for SDS-PAGE.

RESULTS

Effect of prostaglandin A₁ on a cell-free protein-synthesizing system

In order to determine whether PGA₁ interferes directly with the translational machinery, viral mRNAs, isolated from vaccinia virus-infected cells 4-5 h after infection, were translated in a rabbit reticulocyte cell-free system in the presence of different doses (10⁻⁵ to 10 μg/ml) of PGA₁. Control assays received the same amounts of ethanol diluent. Fig. 1 (a) shows that none of the doses of PGA₁ affected the rate of [³⁵S]methionine incorporation into TCA-precipitable material in this system. Different doses of ethanol were shown also not to influence protein synthesis. Moreover, when the polypeptides synthesized in this system were separated by SDS-PAGE (Fig. 1 b), it was found that PGA₁ did not produce any major change in the pattern of the proteins translated, even at doses as high as 10 μg/ml. In spite of the potent effect of PGA₁ on protein synthesis in vaccinia virus-infected cells (Santoro et al., 1982a), these results provided the evidence that PGA₁ did not directly alter protein synthesis in vitro.

Characterization of viral RNAs produced in PGA-treated, vaccinia virus-infected cells

Since we were unable to identify a direct effect of PGA₁ on RNA translation in vitro, we determined whether the inhibition of the synthesis of viral polypeptides that occurs in vivo could be related to changes in the synthesis and/or processing of viral mRNAs. Cytoplasmic RNAs were isolated from untreated and PGA₁-treated (4 μg/ml), uninfected or vaccinia virus-infected cells at 4-5 h after infection, and translated in a cell-free system as described in Methods. Fig. 2 (a) shows that the rate of protein synthesis, measured as incorporation of [³⁵S]methionine in TCA-insoluble material was about threefold higher from RNAs isolated from vaccinia virus-infected cells than in RNAs derived from uninfected cells, as previously reported (Cooper & Moss, 1979). PGA₁ treatment did not cause any difference in the extent of translation of RNAs from uninfected cells but in infected cells, it produced a slight reduction (approx. 20 %) of [³⁵S]methionine incorporation (Fig. 2a). Fig. 2 (b) shows the PAGE analysis of the polypeptides translated in vitro. No significant changes in the synthesis of the polypeptides were noted when different concentrations of RNAs extracted from uninfected L cells (U), treated with PGA₁ (+) or control diluent (−), were used. When RNAs extracted from vaccinia virus-infected cells were used, it was found that, while the general pattern of proteins synthesized was not altered, PGA₁ treatment produced some minor changes in the synthesis of a number of virus proteins and dramatically inhibited the translation of at least three viral RNAs that code for polypeptides
Fig. 1. Effect of different doses of PGA on protein synthesis in vitro. Total cytoplasmic RNA (500 μg/ml) extracted from vaccinia virus-infected mouse L cells at 4.5 h post-infection was translated for 60 min at 30°C in a rabbit reticulocyte cell-free system in the presence of various concentrations of PGA or ethanol diluent. (a) Incorporation of [35S]methionine into acid-insoluble radioactivity. Different concentrations of PGA (O) or the corresponding amount of ethanol diluent (●) were tested. Each point represents the mean of duplicate samples in a representative experiment. Three experiments were carried out with the same results. (b) SDS-polyacrylamide (10 to 15%, w/v) gel electrophoretic analysis of cell-free translational products obtained in the absence (O) or presence of different concentrations of PGA (from 10⁻³ to 10 μg/ml). Molecular weights (× 10⁻³) of some of the vaccinia virus proteins are indicated.

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Fig. 2. Cell-free translation of total cytoplasmic RNA extracted from untreated and PGA₁-treated, vaccinia virus-infected cells. (a) Incorporation of [³⁵S]methionine into acid-insoluble radioactivity in a cell-free system programmed with different concentrations of cytoplasmic RNA incubated at 30 °C for 60 min. ■, Uninfected cells (U); ○, untreated, infected cells (C); ○, prostaglandin-treated, infected cells (P). The extent of incorporation with RNAs (600 µg/ml) from PGA₁-treated uninfected cells was almost identical to control uninfected cells (23,000 ct/min, after 1 h incubation). (b) SDS-PAGE of proteins synthesized in vitro in response to cytoplasmic RNAs from the sources described above. (−), untreated, uninfected; (+), PGA-treated, uninfected. The lesser extent of translation of cellular RNAs as compared to viral RNAs is a consequence of the conditions used (120 mM-K⁺) in the cell-free system optimal for viral translation. Numbers on top of the lanes denote the different concentrations (mg/ml) of RNAs employed. For comparison, the translational products of cytoplasmic RNA from infected HeLa cells are shown: E, early cytoplasmic RNA (600 µg/ml) extracted at 4-5 h post-infection from vaccinia virus-infected HeLa cells in suspension in the presence of cycloheximide (100 µg/ml); L, late cytoplasmic RNA isolated from vaccinia virus-infected HeLa cells in suspension at 4-5 h. Some of the vaccinia virus proteins are indicated to the right of the figure as mol. wt. markers. Arrows indicate those polypeptides whose synthesis was reduced by PGA₁ treatment.

and define whether PGA₁ treatment induced changes in the methylation of RNA, in particular ‘capping’ at the 5’ end, a similar set of experiments was carried out in the presence of S-adenosyl-homocysteine (SAH), an inhibitor of RNA methylation (Banerjee, 1980). In the presence of 200 µM-SAHz, RNAs from PGA₁-treated cells were similarly less effective messengers for protein synthesis (approx. 20% decrease in [³⁵S]methionine incorporation), but there was no difference in the optimal K⁺ concentration required (120 to 140 mM) for both classes of RNA. Moreover, we were unable to switch on the translation of the 38K, 20K and 14K polypeptides by changing ionic concentration in the presence or absence of SAH.

Effect of PGA₁ on the transcription of RNA in vaccinia virus-infected cells

The experiments described above suggest that some viral mRNAs may not be synthesized in PGA-treated, infected cells. In order to verify this hypothesis and to determine the relative
abundance of vaccinia viral RNAs, Southern blot hybridization analysis was performed. After a period of 30 min of virus adsorption, RNAs were labelled with $^{32}$PO$_4$ in the presence or absence of PGA$_1$ (4 µg/ml). $^{32}$P-labelled cytoplasmic RNAs were extracted 4 h later. Early RNAs were obtained by blocking protein synthesis with the addition of 100 µg/ml cycloheximide during the labelling period. Fig. 3 shows the pattern of hybridization of 'early' and 'early + late' classes of viral RNAs to EcoRI restriction fragments of vaccinia DNA, separated by electrophoresis on agarose gel. Although PGA$_1$ did not seem to alter transcription of early RNAs, some major differences were observed in the hybridization to individual restriction fragments when late RNAs from untreated and PGA-treated, infected cells were compared. Whereas some late RNAs were found to hybridize to a similar extent, PGA$_1$ altered the transcription of other late
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Fig. 4. Effect of PGA₁ on RNA polymerase activity in vitro. The kinetics of RNA synthesis was measured by [³H]UTP incorporation into acid-insoluble radioactivity using two systems programmed in vitro either by (a) purified vaccinia virus cores or (b) E. coli RNA polymerase holoenzyme. The concentration of PGA₁ used was 4 µg/ml (○) and controls (●) received the same amount of ethanol diluent.

mRNAs, sometimes decreasing, and sometimes increasing their synthesis, when compared to late RNAs from untreated cells. Moreover, after PGA treatment, at least one RNA band was not present (mol. wt. 1.9 × 10⁶, arrow), and some early RNAs were still synthesized at late times (mol. wt. 2.8 × 10⁶ and 1.3 × 10⁶, dots), a result consistent with the fact that some early viral proteins are synthesized at a late time post-infection in PGA-treated cells (Santoro et al., 1982a). This differential effect of PGA₁ between early and late viral RNAs might suggest a direct action of PGA₁ (possibly by its binding and altering their structure) on some early viral products that regulate the switch-on of later viral genes. However, the possibility that the presence of cycloheximide could inhibit PGA action in vitro should also be considered. Alternatively, PGA₁ could interfere directly with the transcriptional programme of vaccinia virus.

Effect of PGA₁ on transcription of viral mRNA in vitro

In order to establish whether PGA₁ has a direct effect on the transcription of viral RNAs, we tested the effect of different doses of PGA₁ on two in vitro transcription systems, using either the vaccinia virion-associated DNA-dependent RNA polymerase or E. coli RNA polymerase holoenzyme. Fig. 4 shows the time course of [³H]UTP incorporation into acid-insoluble material with both systems, in the presence and absence of 4 µg/ml PGA₁. PGA₁ treatment did not significantly alter the rate of RNA transcription in vitro with either polymerase, thus excluding the possibility that PGA₁ directly interferes with the transcription process. Agarose gel electrophoretic analysis showed that the same sizes of RNAs were produced under each set of conditions (data not shown). Further evidence that PGA₁ does not directly modify viral transcription was obtained in a coupled transcription and translation cell-free system programmed with vaccinia virus cores. Fig. 5 shows that PGA₁ did not change either the rate or pattern of transcription and translation of vaccinia RNAs as determined by kinetic studies and by PAGE analysis of the proteins synthesized in vitro.

DISCUSSION

In this study we have investigated the mechanism of the antiviral action of prostaglandins of the A series in mouse L cells infected with vaccinia virus. Even though very little is known about the molecular basis of the action of prostaglandins, it has been shown that PGs can control ion transport across cell membranes (Lafferty et al., 1972), stimulate cyclic AMP (cAMP) synthesis in cells of different origin (Kuehl, 1974), and trigger synthesis de novo of a cellular
protein (Santoro et al., 1982b). The possibility that changes in the ionic environment of the host cell could influence transcription was excluded after examining cell-free translation of viral RNA in the presence of different concentrations of potassium ions.

The fact that cAMP has an effect opposite to that of PGA₁, enhancing the translation of vaccinia mRNA in a cell-free system through inactivation of the double-stranded RNA form (Legon et al., 1974), and that cAMP does not alter the pattern of proteins synthesized in this system (unpublished results), suggests that the PGA₁ action is not mediated by cAMP. In this report, we have shown that PGA₁ is able to induce major changes in the pattern of RNA transcripts during the course of vaccinia virus infection in L cells and that the previously reported lack of synthesis of some viral polypeptides (Santoro et al., 1982a) in PGA-treated cells correlates with the inhibition of the synthesis of some viral mRNA. These findings were confirmed both by translation in a cell-free system using viral RNA and by Southern blot hybridization of isolated cytoplasmic RNAs, with EcoRI restriction fragments of purified vaccinia DNA. The fact that the majority of the viral RNAs were transcribed and translated with similar efficiencies in both untreated and PGA-treated cells suggests that PGA₁ affects the transcription of some specific viral genes. Since it has been shown (Moss, 1974) that changes in the programmed sequence of events (synthesis of immediate early, early, and then late viral
RNA and proteins) in vaccinia virus replication cause inhibition of virus production, we believe that the changes in the synthesis of RNAs and proteins of vaccinia virus, described in this study, may be responsible for the antiviral action of PGA1.

In conclusion, we have shown for the first time that a prostaglandin can influence the control of gene expression. Since we could not demonstrate a direct effect of PGA1 on transcription in cell-free transcribing systems in vitro, the possibility remains that PGA1 acts through an indirect mechanism. This might be related either to alterations in the host cell, which in turn modify the pattern of viral transcription, or to alterations of the structure and/or function of viral products, which are involved in the switch-on of other viral genes.

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