Prostaglandins of the A Series Inhibit Sendai Virus Replication in Cultured Cells

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SUMMARY

Prostaglandins of the A series (PGA) were shown to be potent inhibitors of Sendai virus replication in African green monkey kidney cells in culture. This antiviral activity was specific for PGA. The effective dose (4 μg/ml) was not toxic to the cells and did not alter either host cell metabolism or infectivity of the virus. PGA did not affect the first stages of virus replication and seemed to act by inhibiting virus maturation and/or budding from the cells. The antiviral action of PGA was pharmacological, was not mediated by cAMP and was completely reversible. Possible mechanisms of action include post-translational binding to virus proteins or interaction with interferon.

INTRODUCTION

The prostaglandins (PGs) are a group of naturally occurring cyclic C20 fatty acids. They are designated by differences in the structure of the cyclopentane ring: series E are β-hydroxyketones, series F are 1,3-diols and series A are α-, β-unsaturated ketones. The native PGs are also characterized as mono-, bi- and tri-unsaturated compounds by the number of C–C double bonds in the aliphatic side chain.

The possibility of a relationship between PGs and virus replication has recently been studied. It has been demonstrated that transformation of Balb/c-3T3 fibroblasts by simian virus 40 (Ritzi & Stylos, 1976) and polyoma virus (Hammarström, 1977) substantially augments PG biosynthesis by these cells. On the other hand, PGs have been recently shown to influence virus replication in cultured cells. Harbour et al. (1978) demonstrated that both PGE2 (10 μg/ml) and PGF2α (1 μg/ml) increased the size of herpes simplex virus (HSV) plaques in Vero cells; PGE2 was also able to increase the yield of virus inoculated at low multiplicity of infection (m.o.i.) but had no effect on the yield of some RNA viruses (measles virus or coxsackie virus B4). However, at higher doses of PGF2α (10 μg/ml), a decrease in virus yield was noted. Luczak et al. (1975) have previously reported that both PGE2 and PGF2α inhibited the multiplication of parainfluenza 3 virus in WISH cells. The authors 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 addition, recent observations have established a relationship between PGs and interferon. Yaron et al. (1977) demonstrated that inducers of interferon stimulated the synthesis of PGs, while Stringfellow (1978) reported that the addition of PGE2, PGA2 and PGF2α (as well as PGE1, PGA1 and PGF1α) restored the interferon response in hyporeactive animals. Moreover, two potent inhibitors of PG synthesis, indomethacin and aspirin, were shown to prevent the establishment of the antiviral state when interferon was added to mouse L cells in the presence...
of vesicular stomatitis virus (Chandrabose et al., 1980). In a recent study (Santoro et al., 1980), we have evaluated the effect of different PGs on the production of Sendai virus by an African green monkey kidney cell (AGMK) line (37RC) in culture. The current manuscript elaborates on the virus-inhibitory effect of PGs of the A series and evaluates the possible mechanisms of this action.

METHODS

Cell cultures. The establishment and the culture technique of 37RC cells have been discussed in detail previously (Benedetto et al., 1979). Briefly, the cells were grown in either T-25 Falcon flasks or in 24-well Linbro plates in Eagle's minimal essential medium (MEM) supplemented with 5% foetal calf serum (Gibco) and antibiotics at 37 °C in a 5% CO₂:95% air, humidified atmosphere. Cells were subcultured by using either 10⁻² M-EDTA (10 min at 37 °C) or trypsin (Difco; 1:250, 0-1%) plus 0-04% EDTA (3 min at room temperature), and re-seeding in fresh medium after washing with phosphate-buffered saline (PBS). This cell line produces interferon upon induction with Newcastle disease virus. Cell counts were performed using a haemocytometer and viability was determined by vital dye (0-02% trypan blue) exclusion.

Virus. Stocks of Sendai virus (obtained from Dr R. Cali6, Institute of Microbiology, University of Rome) were prepared by allantoic inoculation of 10-day-old embryonated eggs with 0-2ml of a 1:1000 dilution of infected allantoic fluid, which was harvested after 72 h at 37 °C, clarified by centrifugation at 4000 g for 10 min and stored at -80 °C.

Virus infection. Confluent monolayers were washed with PBS and then virus samples were added (0.5 ml for Linbro wells and 2 ml for the flasks). After incubation for 1 h at 37 °C, the virus inocula were removed and monolayers were washed three times with PBS and incubated with 1 ml (for Linbro wells) or 10 ml (for the flasks) of MEM. The media were harvested and replaced with fresh MEM daily.

Virus titrations. Virus production was determined by measuring either the haemagglutinating units (HAU) present in the medium or the haemadsorption by infected monolayers of 37RC. Haemagglutinin titrations were done according to standard procedures. For haemadsorption measurements, cell monolayers were washed three times with PBS and 0.5 ml 0.1% human O Rh⁺ red blood cells were added. After incubation at 4 °C for 60 min, monolayers were extensively washed with PBS, and after cell lysis, the number of erythrocytes adsorbed to AGMK cells was quantified by haemoglobin determination using a modification of the technique of Crosby & Furth (1956).

Prostaglandin administration. PGs and synthetic analogues were a generous gift from Dr John Pike, the Upjohn Co., Kalamazoo, Mich., U.S.A. They were stored as 100% ethanolic stock solutions (mg/ml) at -20 °C and were diluted to the appropriate concentrations at the time of their use. Control media contained the identical concentration of ethanol (0-04%). PGs are stable in tissue culture media under the conditions employed and are not further metabolized. Indomethacin and hydrocortisone (Sigma) were also kept as ethanolic stock solutions and diluted identically.

RNA and protein synthesis. For total RNA synthesis, uninfected and virus-infected cell monolayers, in 24-well Linbro plates, were washed twice with 1 ml PBS and 1 ml of fresh medium containing 5 μCi/ml ³H-uridine with and without PGA₁ was added. In some experiments, cells were pretreated for 30 min with actinomycin D (3 μg/ml) before the addition of ³H-uridine, and actinomycin D was maintained in the medium during ³H-uridine incorporation. After 3 h, wells were washed three times with 1 ml sterile 0-9% NaCl, dried, and 1 ml lysis buffer (0-1 M-NaCl, 0-0015 M-MgCl₂, 0-01 M-tris-HCl, 0-5% SDS and 0-5 M-EDTA pH 7) was added. After complete cell detachment, samples were collected and after trichloroacetic acid (TCA) precipitation, the radioactivity was counted in a Beckman
liquid scintillation spectrometer. Total protein synthesis was measured by \(^{35}\)S-methionine incorporation after the cells had been kept for 1 h in methionine-deprived MEM containing 1% dialysed foetal calf serum. After this time, \(^{35}\)S-methionine was added (7.5 \(\mu\)Ci/ml/well) and the cells were incubated for 90 min at 37 °C. Cells were washed three times with 1 ml sterile 0.9% NaCl, dried, and 0.1 ml 0.1 M-NaOH containing 0.1% SDS and 0.1 ml of the lysis buffer were added. After the cells were completely dissolved, samples were collected, vortexed, precipitated with TCA, and the radioactivity counted.

**Statistical analyses.** Statistical analyses were performed using a Student *t*-test for unpaired data. Data were expressed as mean ± s.e.m. and *P* values less than 0.05 were considered significant.

**RESULTS**

**Effect of PGs on Sendai virus production**

Among the different PGs and PG-related compounds tested (PGA\(_1\), PGA\(_2\), PGE\(_1\), PGF\(_{1\alpha}\), prostacyclin, thromboxane B\(_2\), 6-keto PGF\(_{1\alpha}\), arachidonic acid, and two synthetic analogues, 16,16-dimethyl-PGE\(_2\)-methyl ester (di-M-PGE\(_2\)) and 16,16-dimethyl-PGA\(_2\)-methyl ester (di-M-PGA\(_2\)), only the PGs of the A series were noted to be potent inhibitors of Sendai virus production measured by either decreased haemagglutinin titre (HAU) or haemadsorption (HAD) (Santoro *et al.*, 1980). As shown below, PGAs of the 1 and 2 series had identical effects, demonstrating that the number of double bonds in the aliphatic side chains was not critical for the antiviral action. The dose-response effect of the PGAs is shown in Fig. 1. PGA concentrations lower than 0.5 \(\mu\)g/ml had no effect on either haemagglutinin production (Fig. 1a) or haemadsorption (Fig. 1b). At higher concentrations, the inhibition was dose-dependent and reached maximum values at a PGA concentration of 4 \(\mu\)g/ml. Higher concentrations (8 \(\mu\)g/ml) completely inhibited both haemagglutinin production and haemadsorption, but these data were not included, since at this dose, host cell metabolism and growth were slightly altered.

The PGA\(_1\)-mediated inhibition of haemagglutinin production and haemadsorption was independent of the dose of virus used. Confluent monolayers of 37RC cells were infected with Sendai virus at 1 \(\times\) 10\(^4\), 5 \(\times\) 10\(^3\), 1 \(\times\) 10\(^2\) and 1 \(\times\) 10 HAU/2 \(\times\) 10\(^5\) cells. PGA\(_1\) treatment was continued for 48 h. In control cells the production of haemagglutinin was dependent upon the size of the initial inoculum (at 48 h = 64.0 ± 0, 48.0 ± 0, 20.0 ± 0, 5.0 ± 0 and 0 HAU/2 \(\times\) 10\(^5\) cells respectively). PGA\(_1\) treatment produced inhibition which ranged from 86 to 100% of the control, regardless of the dose of the virus inoculum.

In control cells the haemadsorption 48 h after infection was also dependent upon the dose of virus. Inocula containing 1 \(\times\) 10\(^4\), 5 \(\times\) 10\(^3\), 1 \(\times\) 10\(^2\) and 1 \(\times\) 10 HAU/2 \(\times\) 10\(^5\) cells resulted in haemoglobin concentrations of 30.00 ± 1.00, 29.50 ± 2.50, 18.50 ± 2.30, 9.75 ± 0.25 and 5.37 ± 0.37 \(\mu\)g/well respectively.

PGA\(_1\) treatment (4 \(\mu\)g/ml) also inhibited haemadsorption in these cells and the inhibition was independent of the size of the virus inoculum. The percentage inhibition was 70.8, 76.7, 70.3, 73.3 and 84.5% respectively.

**Effect of virus or cell pretreatment with PGA**

To evaluate the possibility that PGA was acting by inactivating the virus itself or by altering cell susceptibility to infection, two different experiments were performed. Stocks of egg-grown Sendai virus were divided into four pools each containing the same number of HAU. They were incubated for 15 min at 37 °C with different concentrations of PGA\(_1\) (0, 1, 10 or 50 \(\mu\)g), then diluted 1:20 in PBS and added to confluent 37RC cell monolayers for 1 h at 37 °C. During this time, the cell infected with PGA-treated virus received either 0.05, 0.5
Fig. 1. Dose-dependent effect of PGA₁ and PGA₂ on Sendai virus production. In this experiment, 24-well Linbro plates were seeded with $1 \times 10^5$ viable 37RC cells; after 48 h, the confluent cell monolayers were infected with egg-grown Sendai at an m.o.i. of $1 \times 10^5$ HAU/10⁶ cells. Treatment with PG was started immediately after infection and continued for 48 h. Media from all cultures were harvested at 24 and 48 h and replaced with fresh media. (a) Release of HAU/2 $\times 10^5$ cells in the medium at 24 h (●—●) and 48 h (O—O). Each point represents the mean of at least two samples. Haemagglutinin titres were identical for each pair of samples at each point (S.E.M. = 0). (b) Haemadsorption measured as μg haemoglobin/well at 48 h after infection with Sendai virus (O—O); ●—●, without virus. At doses exceeding 0.5 μg/mL all PGA samples were significantly different from control ($P < 0.001$).
Fig. 2. Effect of PGA treatment at the late stage of infection. Six 37RC cultures infected with Sendai virus released the same number of HAU into the supernatant 24 h after infection. At this time, medium was harvested and fresh MEM containing either PGA1 (4 µg/ml), PGA2 (4 µg/ml) or ethanol alone (0.04%) was added (arrow). Sendai virus production was measured as (a) HAU released in the medium at 24 and 48 h (○—○, control; •—•, PGA1; ■—■, PGA2) and (b) haemadsorption at 48 h (C, control; IC, infected control; 1, PGA1; 2, PGA2). Each point represents the mean ± S.E.M. of at least duplicate cultures.

PGA treatment was able to suppress virus production even at the late stages of infection. Fig. 2 shows the effect of PGA treatment started 24 h after infection. Both PGA1 and PGA2 were able to suppress both HAU release into the medium (Fig. 2a) and haemadsorption (Fig. 2b).

Effect of PGA1 on host cell metabolism

The effect of PGA1 treatment on both uninfected 37RC cells and Sendai virus-infected cells was studied. Fig. 3(a) shows the effect of different concentrations of PGA1 on uninfected cells. The cells were plated in 4-well Linbro plates at a density of 2 × 10⁴ cells/well. PGA1 treatment was started soon after plating the cells, and medium was changed daily. Doses of PGA1 of 1 or 2 µg/ml did not alter either the rate of cell replication or cell viability (viability always exceeded 96%). The higher dose (8 µg/ml) did not produce any effect at 24 h, while at 48 h, the rate of cell growth was decreased and cell mortality was increased to 7%. For total RNA (Fig. 3b) and protein (Fig. 3c) synthesis, 37RC cells were plated in 4-well Linbro plates and grown to confluence. As soon as confluency was reached (time 0), PGA1 treatment (4 µg/ml) was initiated. Cells were treated for either 24 or 48 h. At these times, RNA and total protein synthesis were measured by ³H-uridine and ³⁵S-methionine incorporation respectively. No difference in either RNA or protein synthesis was detected at this PGA concentration in
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Fig. 3. Effect of PGA₁ treatment on uninfected cells. (a) Growth curve: PGA₁ at 4 μg/ml did not alter growth of cells in separate experiments; (b) RNA synthesis; (c) protein synthesis. PGA₁ (4 μg/ml) was administered at time 0 and maintained in the medium for the subsequent 48 h. (a) ○—○, Control; x—-x, 1 μg/ml PGA₁; O—O, 2 μg/ml PGA₁; △—△, 8 μg/ml PGA₁. (b) and (c) ○—○, control; O—O, PGA₁.

Fig. 4. Effect of PGA₁ treatment on Sendai virus-infected cells. (a) Protein synthesis; (b) RNA synthesis. Treatment was started soon after the 1 h infection period and continued for the subsequent 48 h. ○—○, Control; O—O, PGA₁.

these cells or in Sendai virus-infected cells (Fig. 4). For this latter experiment, 37RC cells were seeded in 4-well Linbro plates at a density of 2 × 10⁵ cells/well and grown to confluency. The monolayers were infected with egg-grown Sendai virus for 1 h at 37 °C (1 × 10⁴ HAU/1 × 10⁶ cells) and PGA₁ treatment (4 μg/ml) was started soon after the 1 h of infection (time 0). Treatment was continued for 24 or 48 h and medium was changed daily. Total protein and RNA synthesis were measured as described in Methods. PGA₁ did not affect either the synthesis of total protein (Fig. 4a) or of RNA (both total and actinomycin D-resistant; Fig. 4b); these data excluded the possibility that PGA₁ acted by inhibiting host cell protein or RNA synthesis.
Antiviral action of PGA

Table 1. Effect of inhibitors of prostaglandin synthesis on Sendai virus replication*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>HAU/2 x 10^5 cells released at</th>
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<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>100, 100†</td>
</tr>
<tr>
<td>Hydrocortisone, 10^-6 M</td>
<td>100, 75</td>
</tr>
<tr>
<td>Indomethacin, 10^-7 M</td>
<td>100, 100</td>
</tr>
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</table>

* Treatment was started soon after the 1 h infection period and continued for the subsequent 48 h. † Pairs of samples were tested.

Table 2. Effect of PGA₁ and dibutyryl-cAMP on HAU release from 37RC cells*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HAU/2 x 10^5 cells released at</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>48, 48</td>
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<tr>
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<td>48, 48</td>
</tr>
<tr>
<td>PGA₁, 4μg/ml</td>
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</tr>
<tr>
<td>Dibutyryl-cAMP, 4 mM</td>
<td>48, 64</td>
</tr>
<tr>
<td>Dibutyryl-cAMP, 2 mM</td>
<td>64, 64</td>
</tr>
<tr>
<td>Dibutyryl-cAMP, 0.5 mM</td>
<td>48, 64</td>
</tr>
<tr>
<td>Dibutyryl-cAMP, 4 mM + PGA₁</td>
<td>2, 3</td>
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</tbody>
</table>

* Treatment was started soon after the infection period and continued for the subsequent 48 h. † Pairs of samples were tested.

Effect of inhibitors of PG synthesis and dibutyryl-cAMP on Sendai virus replication

The effects of two potent inhibitors of endogenous PG synthesis, hydrocortisone and indomethacin, were tested on Sendai virus replication. Hydrocortisone inhibits PG synthesis by blocking the release of the PG precursor, arachidonic acid, from the phospholipid pool within the cell membrane (Santoro et al., 1976; Hong & Levine, 1976), whereas indomethacin is an inhibitor of cyclooxygenase. Table 1 shows that at concentrations known to inhibit PG synthesis in other systems (Santoro et al., 1976) neither of these compounds had a significant effect on HAU release from Sendai virus-infected cells. In this experiment, indomethacin and hydrocortisone were added to the culture media soon after the 1 h infection period and maintained in the medium for the subsequent 48 h. Control cultures received the same amount of ethanol-containing diluent.

The effect of dibutyryl-cAMP, known to stimulate endogenous PG synthesis (Hamprecht et al., 1973), was also investigated. Dibutyryl-cAMP was dissolved in the medium at 0.5, 2 and 4 mM and administered to confluent monolayers of cells soon after the 1 h infection period. Media were changed daily. Table 2 shows the effect of the addition of dibutyryl-cAMP and PGA₁ to Sendai virus-infected cells. Dibutyryl-cAMP at the highest dose augmented the cytopathic effect produced by the virus, but did not significantly alter HAU release from the cells. PGA₁ almost completely inhibited HAU release when used either alone or in the presence of dibutyryl-cAMP. The lack of effect of indomethacin, hydrocortisone and dibutyryl-cAMP suggests that endogenously synthesized PGs are not responsible for the inhibitory action on virus production.

DISCUSSION

In this study, PGAs have been shown to be potent inhibitors of Sendai virus replication in AGMK cells. This antiviral action was very specific for PGAs since no other PG or
The continuous presence of PGA in the medium was necessary for this action and pretreatment of either the cells or the virus before infection did not produce any change in virus replication. PGA administration during the infection period or for short-term (3 h) treatment soon after infection had no effect on virus replication, indicating that PGA did not act during infection or on an early step in the process of virus multiplication. Moreover, we demonstrated that PGAs blocked virus production even in the late stages of infection (24 h), and that long-term treatments could 'cure' infected cells (release of virus in the medium is stopped after 4 days of treatment) and prevent the establishment of a persistent infection (Santoro et al., 1980). Inglot (1969) and Newton (1979) reported that high concentrations of indomethacin and other non-steroidal anti-inflammatory drugs inhibited the replication of a number of viruses. However, at these high doses, indomethacin is also a potent inhibitor of phosphodiesterase activity.

Although two inhibitors of PG synthesis, hydrocortisone and aspirin, were shown to enhance Epstein–Barr virus replication (Magrath et al., 1979) and to increase shedding of rhinovirus (Stanley et al., 1975), we were unable to demonstrate any effect of PG synthesis inhibitors in our system. This, together with the fact that the dose of PGA1 necessary for the antiviral activity was supraphysiological, suggests that the PGA action is pharmacological.

Since, after PGA1 treatment, we were unable to demonstrate any alteration either in the virus RNA (actinomycin D-resistant RNA) or protein synthesis (measured in preliminary experiments by gel autoradiography of 35S-methionine-labelled protein), one possible mechanism of the antiviral action of PGA could be a post-translational modification of a virus protein by PGA1 binding. It is interesting to point out that Schmidt & Schlesinger (1979) have recently demonstrated that the glycoprotein (G) of vesicular stomatitis virus bound 1 to 2 mol fatty acid/mol protein. This binding was a post-translational event; it seemed to occur as G protein reached the cell surface and to be associated with maturation of G protein. It could be speculated that PGA might take the place of the physiological fatty acid, competing for binding to a virus protein and thus inhibit virus maturation. This possibility is currently being studied.

However, another interesting possibility is that the antiviral effect was mediated by interferon. As discussed in Introduction, interferon has been shown to stimulate the synthesis of PGs (Yaron et al., 1977), and PGs of the E, A and F series were shown to restore the interferon response in hyporeactive animals (Stringfellow, 1978). The fact that in preliminary experiments, PGA1 produced much less inhibition of Sendai virus replication in a non-interferon-producing cell line (Vero cells) seems to support this hypothesis. However, further study is necessary to evaluate the mechanism of the antiviral action of PGA and the possibility of using their ability to control virus replication in vivo, as well as in cultured cells.

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REFERENCES


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