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Inhibition of Poliovirus Replication by Prostaglandins A and J in Human Cells

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Cyclopentenone prostaglandins (PGs) inhibit the replication of a wide variety of enveloped DNA and RNA viruses. The antiviral activity is associated with alterations in the synthesis, maturation, and intracellular translocation of viral proteins. In the present report, we describe the effects of cyclopentenone PGs PGA_1 and Δ^{12}-PGJ_2 on poliovirus (PV) replication in HeLa cells. Both PGs were found to inhibit PV replication dose dependently. Virus yield was significantly reduced at nontoxic concentrations, which did not suppress RNA or protein synthesis in uninfected or PV-infected cells. Both the pattern of PV proteins synthesized and the kinetics of viral protein synthesis and degradation appeared to be similar in PGA_1-treated cells and control cells. Antiviral PGs have been shown to selectively inhibit virus protein synthesis during the replication of several viruses, including vesicular stomatitis virus (VSV), and this effect has been recently associated with the induction of a 70-kDa heat shock protein (HSP70). PGA_1 and Δ^{12}-PGJ_2 were found to induce HSP70 synthesis in uninfected or VSV-infected HeLa cells. PV infection was found to inhibit PG-induced HSP70 synthesis in these cells, suggesting that the lack of ability of cyclopentenone PGs to block PV protein synthesis could be related to an impaired heat shock response in PV-infected cells. The finding that PV protein synthesis was not inhibited by PGs suggests that cyclopentenone PGs could interfere with a late event in the virus replication cycle, such as protein assembly and maturation of PV virions.

Poliovirus (PV), an enterovirus within the family *Picornaviridae*, is a small, positive-strand RNA virus which replicates in the human oropharynx and lower gastrointestinal tract, extending to the central nervous system in 1 to 4% of cases (15). Within the host cell, the viral RNA is translated into a single polyprotein, which is then processed into functional structural and nonstructural proteins by the virus-encoded proteases 2A and 3CD (24). An RNA-dependent RNA polymerase encoded by the viral genome is necessary for the synthesis of a complementary negative-strand RNA, which in turn serves as a template for additional positive-strand copies of the genome. As the structural viral proteins accumulate, increasing amounts of viral RNA are encapsidated into mature virions, which are released as the host cell is destroyed (15).

Prostaglandins (PGs) of the A and J types, characterized by the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring (cyclopentenone PGs), have been shown to inhibit the replication of a wide variety of enveloped DNA and RNA viruses, including human immunodeficiency virus type 1 (reviewed in reference 16). Even though in the last decade major advances in the identification of the cellular and viral targets of PGs have been made, the mechanism of the antiviral activity is complex and has not been yet completely elucidated. In the case of negative-strand RNA viruses, it has been demonstrated that cyclopentenone PGs affect two separate events of virus replication, at an early and a late phase of the virus cycle, and can alter the synthesis and/or maturation of viral proteins (16). The antiviral activity has been recently associated with the induction of heat shock protein synthesis, in particular of the 70-kDa heat shock protein (HSP70) (1, 14, 16).

In the present study, we have examined the effects of PGA_1 and Δ^{12}-PGJ_2 (9-deoxy-Δ^9,Δ^{12}-13,14 dihydro-PGD_2), a natural dehydration product of PGD_2 physiologically present in human body fluids (5, 7), on the multiplication of a positive-strand RNA virus, PV, and on the induction of HSP70 in HeLa cells.

MATERIALS AND METHODS

Cell cultures. HeLa (Ohio) cells were grown at 37°C in a 5% CO_2 atmosphere in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU of penicillin G per ml, and 100 μg of streptomycin per ml. Cells were counted in a hemocytometer, and cell viability was determined by the dye exclusion technique, as described previously (22).

Virus infection and titration. Confluent HeLa cell monolayers were infected with PV type 2 P712 (5 PFU per cell) or with vesicular stomatitis virus (VSV) (Indiana strain) (5 PFU per cell). After 1 h of adsorption at 37°C, the viral inoculum was removed, and the cell monolayers were washed three times with phosphate-buffered saline (PBS) and incubated with 1 ml of MEM containing 2% FCS. PGA_1 and Δ^{12}-PGJ_2 (Cayman Chemical Co.) were stored in absolute ethanol and diluted to the appropriate concentration at the time of use. Control media contained the same concentration of ethanol diluent, which was shown not to affect cell metabolism or virus replication.

Virus production was determined by plaque assay. After the infected cells were frozen and thawed three times, serial 10-fold dilutions of PV or VSV were inoculated on confluent HeLa cell monolayers. After 1 h at 37°C, the inoculum was removed, and the cells were washed three times with PBS before the addition of MEM containing 2% FCS and 1% SeaPlaque agarose (Miles). After 2 days of incubation at 37°C in a 5% CO_2 atmosphere, plaques were stained with 0.33% neutral red solution.

DNA, RNA, and protein synthesis. Confluent monolayers of uninfected or virus-infected HeLa cells (5 PFU per cell) were labeled for 12 h, starting soon after virus infection, with 5 μCi of [^{3}H]thymidine, [^{3}H]uridine, or [^{35}S]methionine (Amersham International) per ml per 5×10^5 cells for DNA, RNA, or protein synthesis, respectively, and the radioactivity incorporated into acid-soluble and -insoluble material was determined as described previously (17).

Protein synthesis and SDS-PAGE analysis. Confluent cell monolayers were labeled with [^{35}S]methionine (15 min; 1-, 3-, or 8-h pulses; 5 μCi/ml/5×10^5 cells) in methionine-free medium containing 2% dialyzed FCS. The cells were usually

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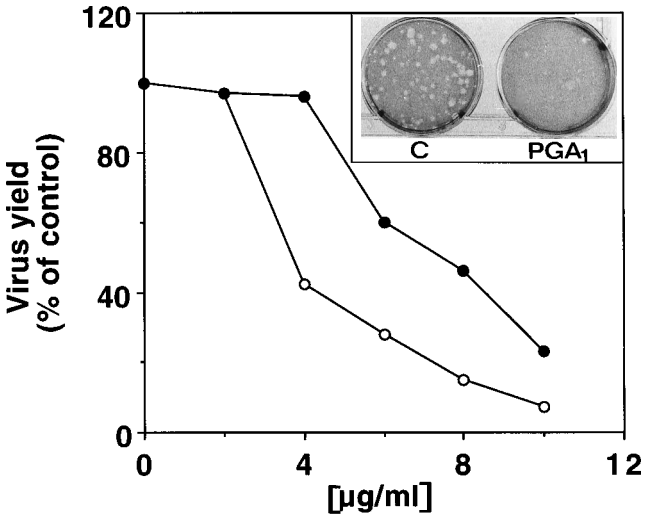

FIG. 1. Dose-dependent inhibition of PV replication by PGA_1 and Δ^{12}-PGJ_2. Confluent monolayers of HeLa cells were infected with PV (5 PFU per cell) for 1 h at 37°C. Soon after infection, culture medium containing PGA_1 (●), Δ^{12}-PGJ_2 (○), or control diluent was added. Virus production was determined by plaque assay at 8 hpi. Each point represents the mean of duplicate samples. Each experiment was repeated three times with similar results. (Inset) Reduction of PV plaque size and number by PGA_1 (10 μg/ml) added directly to the agar overlay during plaque assay. Approximately 100 plaques were measured in triplicate cultures for each sample. C, control.

preincubated for 15 min in methionine-free medium. After being labeled, the cells were washed and lysed in lysis buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.001% bromophenol blue, 0.1 M dithiothreitol, 0.0625 M Tris-HCl; pH 6.8) and the radioactivity incorporated into trichloroacetic acid-insoluble material was determined. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a vertical slab gel apparatus (3% stacking gel, 10 or 12% resolving gel) and processed for autoradiography and densitometric analysis, as described previously (1).

Immunoblot analysis. For immunoblot analysis, an equal amount of protein from each sample was separated by SDS-PAGE and blotted onto nitrocellulose, as described previously (1). After transfer, the filters were incubated with an anti-72/73-kDa heat shock protein monoclonal antibody (diluted 1:500) from HeLa cells (Amersham) in Ten-Tween 20 buffer (0.05 M Tris-HCl [pH 7.4], 5 mM EDTA, 0.15 M NaCl, 0.05% Tween 20), and the bound antibody was detected by using horseradish peroxidase-linked sheep anti-mouse antibody (Amersham International). Molecular weights were calculated by using Bio-Rad low-M_r markers.

Statistical analysis. Statistical analyses were performed by using the Student *t* test for unpaired data. Data are expressed as the means ± standard deviations of at least two samples; *P* values of <0.05 were considered significant.

RESULTS

Effects of PGs on PV replication. The effects of PGA_1 and Δ^{12}-PGJ_2 on PV infection of HeLa cells have been studied under one-step multiplication conditions. After the 1-h adsorption period, different concentrations of PGA_1, Δ^{12}-PGJ_2, or control diluent were added to the culture medium and the viral yield was determined by plaque assay 8 h postinfection (hpi). As shown in Fig. 1, both PGs were found to reduce PV production dose dependently, and an inhibition of approximately 80% was obtained at a concentration of 10 μg of PGA_1 per ml or 6 μg of Δ^{12}-PGJ_2 per ml. Higher concentrations of either PG could totally suppress virus replication but were found to inhibit host cell macromolecular synthesis (data not shown). In a different type of experiment, HeLa cell monolayers were infected with PV (100 PFU per dish), and, after 1 h at 37°C, the viral inoculum was removed and medium containing 2% FCS, 1% agarose, and PGA_1 (10 μg/ml) or control diluent was added. At 48 hpi, the number and the size of plaques were determined after neutral red staining. Also under these conditions, PGA_1 treatment inhibited PV replication and caused a dramatic reduction in both the number of plaques (>90% inhibition compared with the control) and the plaque size (Fig. 1, inset). The average diameter of PV plaques was decreased by approximately 75% in PG-treated cells versus control untreated cells. Similar effects were produced by treatment with Δ^{12}-PGJ_2 at concentrations above 4 μg/ml (data not shown).

Under the conditions described above, both PGs were shown not to be toxic to uninfected cultures, as determined by microscopic examination and vital dye uptake. The effects of PGA_1 or Δ^{12}-PGJ_2 treatment on DNA, RNA, and protein synthesis in uninfected or PV-infected HeLa cells were determined after labeling with [^{3}H]thymidine, [^{3}H]uridine, or [^{35}S]methionine, respectively, as described in Materials and Methods. In uninfected cells, a 12-h treatment with PGA_1 (10 μg/ml) did not inhibit DNA and protein synthesis, while it moderately increased RNA synthesis. Since PGA_1 is known to inhibit tumor cell proliferation (6), the effect of PGA_1 on HeLa cell growth was also investigated. When HeLa cells (10^5) were seeded in the presence of PGA_1 (10 μg/ml) or ethanol diluent, PGA_1 only transiently (24 h) inhibited cell proliferation, and no significant difference in the number of cells in PGA_1-treated or -untreated cultures was found 48 h after treatment [control, $(4.8 \pm 0.3) \times 10^5$ cells per ml; PGA_1, $(4.3 \pm 0.3) \times 10^5$ cells per ml]. Δ^{12}-PGJ_2 (6 μg/ml) was also found not to alter RNA and protein synthesis, while it only moderately inhibited DNA synthesis. As expected, PV infection greatly reduced DNA and RNA synthesis in HeLa cells. Treatment with either PGA_1 (10 μg/ml) or Δ^{12}-PGJ_2 (6 μg/ml) did not significantly alter macromolecular synthesis or uptake of precursors in PV-infected cells (data not shown).

Effects of cyclopentenone PGs on host cell and PV protein synthesis. To determine the effect of PG treatment on PV protein synthesis, confluent HeLa cell monolayers were infected with PV and were treated with PGA_1 (10 μg/ml), Δ^{12}-PGJ_2 (6 μg/ml), or control diluent after the 1-h adsorption period. The cells were labeled with [^{35}S]methionine either soon after infection for the following 8 h (Fig. 2A) or 5 h after PV infection for the next 3 h (Fig. 2B). Uninfected cells were treated identically. Cell extracts were separated by SDS-PAGE and processed for autoradiography. In uninfected HeLa cells, treatment with PGA_1 and Δ^{12}-PGJ_2 did not greatly alter the overall electrophoretic profile of cellular proteins, but it decreased the synthesis of a 96-kDa protein and induced the synthesis of a 72-kDa protein (Fig. 2A and B). Δ^{12}-PGJ_2 was found to be much more effective an inducer than PGA_1. This protein was identified as a heat shock protein related to the major HSP70 group by immunoblot analysis (Fig. 2C). It should be pointed out that elevated constitutive levels of HSP70 were detected in untreated HeLa cells by immunoblot analysis (Fig. 2C). In PV-infected cells, host cellular protein synthesis was dramatically inhibited 5 hpi. Under the conditions described above, PV protein synthesis was not affected by PGA_1 and only slightly inhibited by Δ^{12}-PGJ_2. Interestingly, differently from the results previously obtained during infection with other types of RNA viruses, including VSV and Sendai virus (1, 14), infection with PV prevented HSP70 induction by PGA_1 and inhibited HSP70 induction by Δ^{12}-PGJ_2 (Fig. 2A, B, and D).

To study the effect of PGA_1 treatment on the kinetics of PV protein synthesis, HeLa cell monolayers infected with PV were treated with PGA_1 (10 μg/ml) or control diluent soon after the 1-h adsorption period and were labeled with [^{35}S]methionine (1-h pulse) at different intervals from 1 to 8 hpi. As shown in Fig. 3), PV infection resulted in the expected inhibition of host

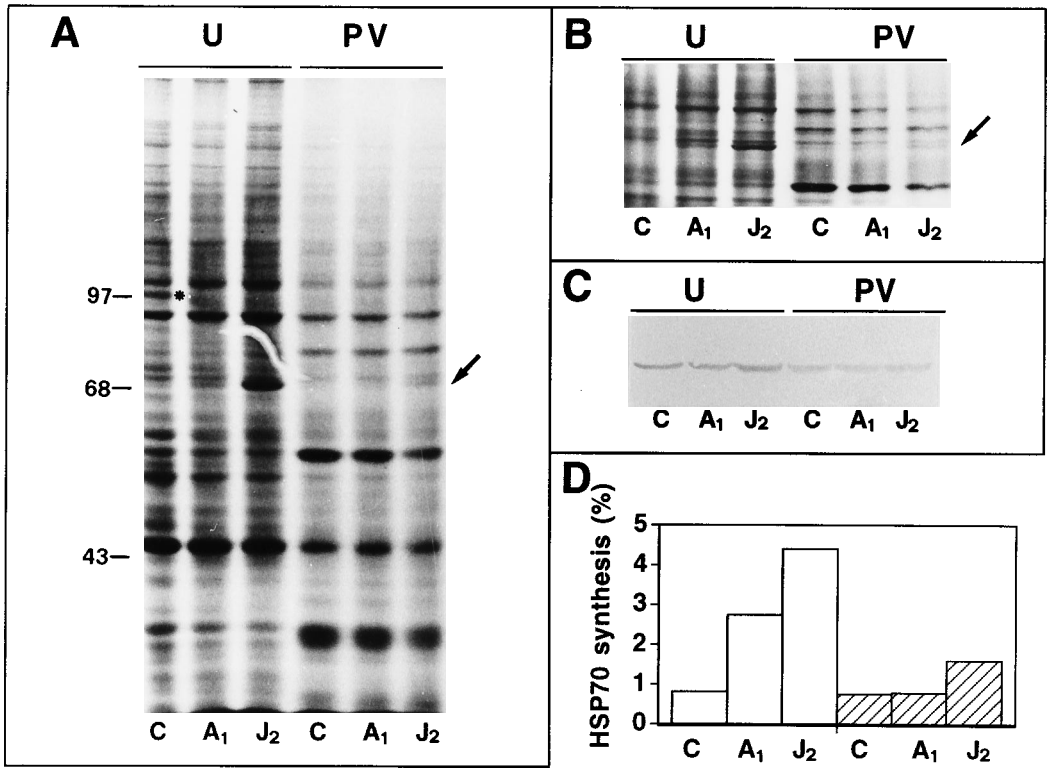

FIG. 2. Effect of PG treatment on protein synthesis in uninfected and PV-infected HeLa cells. Results of SDS-PAGE analysis and autoradiography of polypeptides synthesized in uninfected (U) or PV-infected (PV) HeLa cells, treated with control diluent (C), 10 μg of PGA_1 per ml (A_1), or 6 μg of Δ^{12}-PGJ_2 per ml (J_2), for 8 h after the 1-h adsorption period, are shown. The cells were labeled with [^{35}S]methionine (5 μCi per well, 8- or 3-h pulse), starting soon after (A) or 5 h after (B) the beginning of PG treatment. Samples containing equal amounts of radioactivity were separated by SDS-PAGE (10% polyacrylamide resolving gel) and processed for autoradiography. The positions of the 72-kDa protein induced in PG-treated cells (arrows) and of a 96-kDa protein whose synthesis is inhibited by PGA_1 (asterisk) are indicated. Molecular masses (in kilodaltons) are shown on the left. (C) Identification of HSP70 by immunoblot analysis using anti-human HSP70 monoclonal antibodies in the samples described in panel A. (D) HSP70 synthesis in samples in panel A was quantified by densitometric analysis and expressed as a percent of total protein synthesis. □, uninfected cells; ▨, PV-infected cells.

cell translation (15) and in the synthesis of virus-specific proteins starting after 2 hpi. The pattern and the time course of PV protein synthesis in PGA_1-treated cells appeared to be similar to those of untreated control cells (Fig. 3). Densitometric analysis of the autoradiographic patterns revealed only a modest (10 to 15%) reduction of the synthesis of viral proteins in PGA_1-treated cultures compared with that of the control, indicating that the translation of the virus messages is not the major target for PGA_1 antiviral activity. It should be noted that while HSP70 synthesis could not be detected in PV-infected cells, the synthesis of an as yet unrecognized 68-kDa protein, characterized by an electrophoretic mobility (~4 kDa) different from that of HSP70, appeared to be enhanced in PGA_1-treated cells 6 to 8 h after PV infection.

To investigate whether PGA_1 was affecting PV protein stability, HeLa cells were infected with PV and then treated with PGA_1 soon after the 1-h adsorption period. At 5 h after infection, cells were washed, incubated for 15 min in medium lacking methionine, and then incubated for 15 min in the same medium supplemented with [^{35}S]methionine (50 μCi/10^5 cells). After this time, the cells were washed three times with MEM supplemented with 10 times the normal concentration of methionine and chased for different times. As shown in Fig. 4, PGA_1 did not appear to interfere with PV protein stability, suggesting an effect on virion assembly or maturation.

Effect of PGA_1 on VSV replication in HeLa cells. It has been previously shown that PGA_1 treatment causes a selective block of virus protein synthesis in different types of monkey epithelial cells infected with negative-strand RNA viruses (1, 14). This block has been associated with PGA_1-mediated induction of HSP70 synthesis. In order to determine whether the different effect of PGA_1 described above could be attributed to a different response of HeLa cells to PGs or whether it was dependent on the type of virus infection, confluent monolayers of HeLa cells were infected with VSV (5 PFU per cell) and treated with PGA_1 at different concentrations. Virus yields were determined 8 h after infection. PGA_1 effectively inhibited VSV replication in HeLa cells, and an inhibition of >95% of the control value was found at the concentration of 10 μg/ml (Fig. 5A). To determine the effect of PGA_1 on protein synthesis and HSP70 induction in VSV-infected cells, HeLa cells infected with VSV (1 PFU per cell) were treated with 10 μg of PGA_1 per ml after the 1-h adsorption period and labeled with [^{35}S]methionine 6 h after infection. As shown in Fig. 5C, PGA_1 was found to dramatically inhibit VSV protein synthesis in HeLa cells, as previously reported for other cell lines (14). Moreover, VSV infection was not able to prevent HSP70 induction by PGA_1 in HeLa cells, and no difference in HSP70 synthesis was found in VSV-infected cells in comparison with uninfected cells (Fig. 5B).

DISCUSSION

PGs of the A and J types possess a potent antiviral activity against several DNA and RNA viruses, including poxviruses

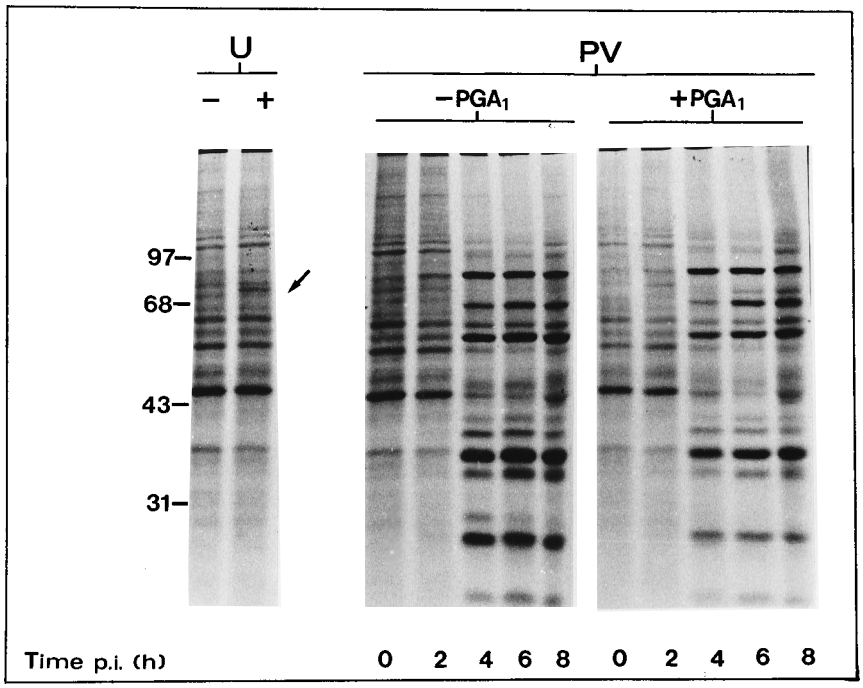

FIG. 3. Effect of PGA_1 on the kinetics of PV protein synthesis in HeLa cells. Results of SDS-PAGE analysis and autoradiography of proteins from PV-infected HeLa cells (PV) treated with ethanol diluent ($-PGA_1$) or 10 μg of PGA_1 per ml ($+PGA_1$) are shown. Cells were labeled (1-h pulses) with 5 μCi of [^{35}S]methionine per ml at different times postinfection (p.i.). Samples containing equal amounts of radioactivity were separated by SDS-PAGE (12% resolving gel) and processed for autoradiography. The position of HSP70 induced by PGA_1 (arrow) in uninfected (U) HeLa cells, labeled with [^{35}S]methionine (1-h pulse) starting 4 h after PG treatment, is indicated. Molecular masses (in kilodaltons) are indicated on the left.

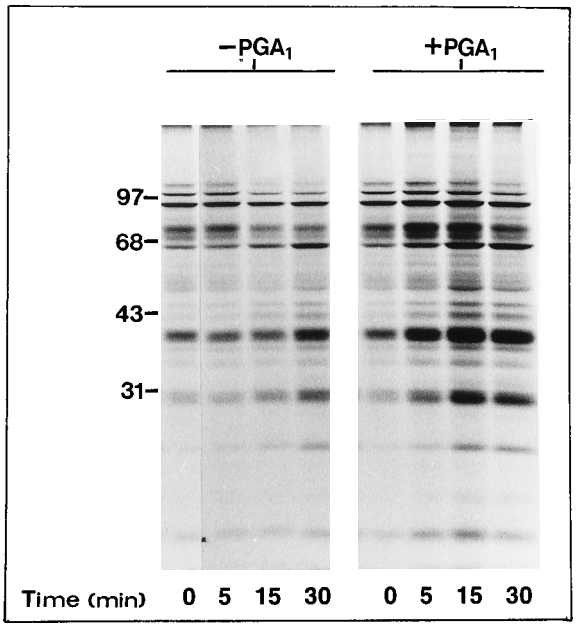

FIG. 4. Pulse-chase analysis of PV proteins in PV-infected HeLa cells treated with PGA_1. Confluent monolayers of HeLa cells infected with PV (5 PFU per cell) were treated with PGA_1 ($+PGA_1$) or ethanol diluent ($-PGA_1$) after the 1-h adsorption period and labeled with [^{35}S]methionine (50 μCi/10^5 cells, 15-min pulse) at 5 hpi. After washing, the cells were chased for different times in MEM containing 10× unlabeled methionine. Samples containing the same amounts of protein were processed for SDS-PAGE (12% resolving gel) analysis and autoradiography. Molecular masses (in kilodaltons) are shown on the left.

(21), herpesviruses (8, 25), paramyxoviruses (1, 18), orthomyxoviruses (19), rhabdoviruses (20), togaviruses (11), and retroviruses (4, 8). Inhibition of encephalomyocarditis virus replication in murine cells has also been reported (3).

The mechanism of the antiviral activity has been studied in detail in two models of negative-strand RNA viruses, VSV (14) and Sendai virus (1). In both cases, it has been shown that cyclopentenone PGs can inhibit virus replication at two separate levels during the virus replication cycle. Cyclopentenone PGs specifically alter the glycosylation and intracellular transport of HN and F glycoproteins during the replication of Sendai virus (17) and affect G-glycoprotein maturation in VSV-infected mammalian cells (14, 20). Moreover, cyclopentenone PGs have been shown to selectively block VSV and Sendai virus protein synthesis and to protect the host cell from the virus-induced shutoff of cellular protein synthesis (1, 14). This block is exerted at the translational level and is associated with the induction of HSP70 (1, 16).

Heat shock proteins are a group of polypeptides whose synthesis represents a finely regulated response of both prokaryotic and eukaryotic cells to adverse environmental conditions, including heat shock and virus infection (9, 16). In eukaryotic cells, heat shock proteins are generally present as multigene families, consisting of closely related protein isoforms, with some members expressed constitutively and others expressed after exposure to environmental stress. The constitutive HSP70 proteins are needed during protein synthesis for protein folding, assembly, and intracellular translocation (9, 12).

Constitutive HSP70 was shown to be associated with newly synthesized capsid precursor P1 of PV, and the HSP70-P1 complex was found to be part of an assembly intermediate of PVs, rather than of the mature virion, suggesting a functional role for HSP70 in virus assembly (10). It should be pointed out

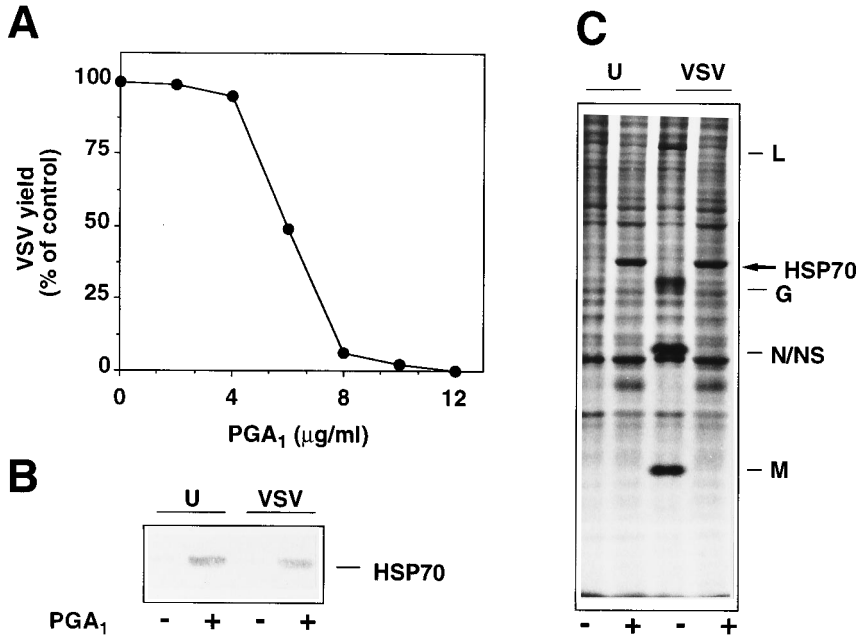

FIG. 5. Effect of PGA_1 on VSV replication and HSP70 synthesis in HeLa cells. (A) Confluent HeLa cell monolayers were infected with VSV and treated with different concentrations of PGA_1 after the 1-h adsorption period. Virus yield was determined at 8 hpi. For each point, the data are the means of duplicate samples. Standard deviations of duplicate samples were <10%. (B and C) Uninfected (U) or VSV-infected (VSV) HeLa cells were treated with PGA_1 (10 μg/ml) (lanes +) or ethanol diluent (lanes −) after the 1-h adsorption period and labeled with [^{35}S]methionine (1-h pulse) 6 h after infection. Samples containing equal amounts of protein were processed for immunoblot analysis, using anti-human HSP70 monoclonal antibodies (B). Samples containing equal amounts of radioactivity were processed for SDS-PAGE analysis (10% resolving gel) and autoradiography (C). HSP70 and VSV proteins L, G, N, NS, and M are indicated.

that PV infection was shown to inhibit constitutive (10) or heat shock-induced (13) HSP70 synthesis 2 to 3 h after infection, even though in the latter case, synthesis of heat shock proteins was found to be more resistant to inhibition than that of normal host proteins after PV infection (13). On the other hand, the translation of the glucose-regulated protein BiP was found to be increased in PV-infected HeLa cells, at a time when cap-dependent translation of cellular mRNA is inhibited (23).

In the present report, we show that cyclopentenone PGs inhibit PV replication. The reduction of PV yield is dose dependent. A significant inhibition of PV production has been observed concentrations of PGA_1 and Δ^{12}-PGJ_2 which did not inhibit nucleic acid and protein synthesis in both uninfected and infected HeLa cells. In the presence of PG concentrations which inhibited virus production, both the pattern of the PV proteins synthesized and the kinetics of synthesis appeared to be similar to those of the control. Also, the stability of PV proteins did not appear to be altered by PGA_1 treatment.

Since cyclopentenone PGs function as signals for HSP70 induction in a large variety of mammalian cells (2, 16, 22), we have studied the effects of PGA_1 and Δ^{12}-PGJ_2 on HSP70 synthesis in uninfected and PV-infected HeLa cells. Both PGA_1 and Δ^{12}-PGJ_2 were found to induce HSP70 synthesis in HeLa cells, and high intracellular levels of HSP70 were found to be accumulated in uninfected cells 8 h after PG treatment. As previously shown for heat shock-treated cells (13), infection with PV was found to inhibit HSP70 synthesis in PGA_1- and in Δ^{12}-PGJ_2-treated HeLa cells. In this respect, PV appears to behave differently from the other RNA viruses previously studied, which did not interfere with PG-induced HSP70 synthesis (1, 14). This effect could be due to the dramatic shutoff of cellular protein synthesis caused by PV, which is known to rapidly inhibit cap-dependent translation of cellular mRNA (15). We therefore compared HSP70 synthesis after treatment with PGA_1 in HeLa cells infected with PV or with VSV. VSV infection did not influence the heat shock response in HeLa cells; moreover, HSP70 induction was associated with a more efficient inhibition of VSV replication and with a dramatic reduction of VSV protein synthesis, as previously shown for different cell lines (14). These results suggest that the lack of ability of cyclopentenone PGs to block PV protein synthesis, in contrast to the results previously described for different viral models, could be due to an impaired heat shock response in cells infected with PV. The finding that considerable amounts of PV proteins were synthesized at concentrations which suppressed virus production by more than 90% suggests that cyclopentenone PGs could interfere with a late event of the virus replication cycle, such as protein assembly and maturation of PV virions.

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