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Inhibition of Sendai virus replication by Δ^{12}-prostaglandin J_2: induction of heat shock protein synthesis and alteration of protein glycosylation

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Summary

Δ^{12}-Prostaglandin J_2 (Δ^{12}-PGJ_2), a natural dehydration product of prostaglandin D_2 present in human body fluids, was shown to suppress Sendai virus replication in monkey kidney cells, at doses non-toxic to uninfected cells. Dramatic inhibition of virus production could be obtained at doses of Δ^{12}-PGJ_2 which did not inhibit cellular or viral protein synthesis, suggesting an effect on virus assembly and/or maturation. At the active concentration, Δ^{12}-PGJ_2 caused a decrease in glucosamine incorporation into the virus glycoproteins HN and F, and in at least one host cell polypeptide, while it did not affect most cellular glycoproteins and it induced the glycosylation of a 47-kDa cellular polypeptide. These effects were accompanied by the induction of heat shock protein synthesis, which was found to differ in its specificity and kinetics from induction by prostaglandin A_1.

Prostaglandin; Sendai virus; Cyclopentenone; Heat shock protein; Glycosylation

The antiviral activity of prostaglandins of the A type (PGAs) was first reported in 1980 (Santoro et al., 1980). Starting from this early observation, several studies have shown that PGAs can suppress the replication of a large number of RNA and DNA viruses, among which orthomyxoviruses,

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paramyxoviruses, picornaviruses, rhabdoviruses, togaviruses, poxviruses, herpesviruses and retroviruses, independently of the type of host cells (reviewed in Santoro, 1987; Santoro et al., 1990). The antiviral activity of a long-acting synthetic analog of PGA_2 has also been shown in vivo, in a mouse model infected with influenza A virus (Santoro et al., 1988).

In all models studied, the antiviral activity is associated with alterations in the synthesis and/or maturation of specific virus proteins, apparently through a cell-mediated mechanism (Santoro, 1987; Santoro et al., 1987, 1989a). Among several types of prostaglandins tested only PGAs and PGJ_2 were found to be potent inhibitors of virus replication at doses non-toxic to the host cells. PGAs and PGJs are characterized by the presence of an α,β-unsaturated carbonyl group in the cyclopentane ring of the molecule (cyclopentenone PGs), which could be the necessary feature for the antiviral activity to occur.

In the present report, we describe the antiviral activity of Δ^{12}-PGJ_2 (9-deoxy-Δ^9,Δ^{12}-13,14-dihydro-PGD_2), a natural dehydration product of PGD_2 present in human body fluids (Hirata et al., 1988), in monkey kidney cells infected with Sendai virus (SV). Δ^{12}-PGJ_2 appears to be the ultimate natural metabolite of PGD_2 (Fukushima, 1990). Even though it has been suggested that Δ^{12}-PGJ_2 may derive from PGJ_2 (9-deoxy-Δ^{12}-PGD_2), the biological activities of these two compounds are not identical, as has been shown for activity on platelet aggregation (reviewed in Fukushima, 1990). The results indicate that Δ^{12}-PGJ_2 is a potent inhibitor of Sendai virus replication and that this effect is associated with the induction of a set of cellular proteins, identified as heat shock proteins, and with alterations of protein glycosylation.

African green monkey kidney cells (37RC cell line) were grown in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS; Gibco) and antibiotics, at 37°C in a 5% CO_2 atmosphere. PGA_1 and Δ^{12}-PGJ_2 (Cayman Co.) were stored as 100% ethanolic stock solutions (2 mg/ml) and tested at the concentration of 4 μg/ml unless otherwise specified. Control medium contained the same concentration of ethanol (0.02%) which did not affect cell metabolism or virus replication. Confluent cell monolayers were infected with SV (10 HAU/2 $\times$ 10^5 cells), prepared by allantoic inoculation of embryonated eggs (Santoro et al., 1989a). After incubation for 1 h at 37°C, virus inocula were removed and monolayers were washed three times with PBS and incubated with fresh medium containing 2% FCS. Virus production was determined by measuring the hemoagglutinin units (HAU) present in the medium or the hemoglobin content of the erythrocytes adsorbed on infected monolayers (HAD), as previously described (Santoro et al., 1980). Statistical analyses were performed using Student's t-test for unpaired data and P-values of < 0.05 were considered significant. For HAU titres, S.E.M. = 0 indicated that titres were identical for each pair of samples.

For protein synthesis studies, confluent cell monolayers were labeled with [^{35}S]methionine (5 μCi/2 $\times$ 10^5 cells) in methionine-deprived medium, containing 2% dialyzed fetal calf serum. After labeling, cells were washed, lysed in lysis buffer (2% SDS, 10% glycerol, 0.001% bromophenol blue, 0.1 M

dithiothreitol, 0.0625 M Tris-HCl pH 6.8) and, after determining the radioactivity incorporated, samples were analyzed by SDS-PAGE in a vertical slab gel apparatus (3% stacking gel, 7.5% resolving gel, unless differently specified) and autoradiographed as previously described (Santoro et al., 1989a). M_r values of polypeptides were calculated using the following protein mixture: phosphorylase b (97-kDa), bovine serum albumin (68-kDa), ovalbumin (43-kDa), carbonic anhydrase (31-kDa), lysozyme (14-kDa). Virus proteins were identified by viral markers P, HN, NP, F and M from unlabeled egg-grown purified Sendai virus. For immunoblot analysis, equal amounts of protein for each sample were separated by SDS-PAGE as described above, and blotted to nitrocellulose using the technique described by Burnette (1981). After transfer, the filters were incubated with a monoclonal anti-72/73-kDa HSP antibody (MAb, 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 horseradish peroxidase-linked sheep anti-mouse antibody (Amersham). Molecular masses were calculated using BioRad low M_r (LMW) markers. Quantitative determination of protein synthesis has been measured by densitometric analysis of autoradiographed patterns using a laser-beam densitometer (Ultroscan XL, LKB). Alternatively, equal amounts of [^{35}S]methionine-labelled proteins were separated by SDS-PAGE and processed for immunoblot analysis. After immunodetection, the individual bands were excised from the blots and the radioactivity incorporated was measured in a β-scintillation counter, as previously described (Amici and Santoro, 1991).

The cytopathic effect (CPE) produced by Sendai virus starts to be evident approximately 12 h post-infection (p.i.); by 72 h p.i., most cells will detach from culture plates and die. Treatment with Δ^{12}-PGJ_2, started after the 1-h infection period, did not prevent SV-induced CPE, but generally delayed its appearance for 12–24 h, and dramatically suppressed virus production by infected cells. Δ^{12}-PGJ_2 antiviral activity was dose-dependent, with a selective index of 16 (Fig. 1a). The concentration of 4 μg/ml was the most active dose (inhibiting virus production by 90–100% in 4 separate experiments), which did not inhibit protein synthesis in uninfected cells. A single treatment with Δ^{12}-PGJ_2 (4 μg/ml), started after the 1-h infection period, suppressed virus replication by more than 90% at all times p.i. (Fig. 1b). Δ^{12}-PGJ_2 did not act by inactivating the virus itself or by inhibiting virus adsorption by the cells. Neither SV-pretreatment with Δ^{12}-PGJ_2 (4 μg/ml) for 15 min (Fig 1c,d) or 90 min (data not shown) before infection, nor its presence during the 1-h infection period had any effect on virus replication (Fig. 1c,d).

We have previously shown that both the PGA_1- and PGJ_2-induced block of SV replication is mediated by alterations in the synthesis of specific viral proteins and is accompanied by the induction of the synthesis of a 74-kDa polypeptide (Santoro et al., 1987, 1989a) identified as a heat shock protein (HSP) of the HSP70 family (Amici and Santoro, 1991). To investigate the effect of Δ^{12}-PGJ_2 on cellular and viral protein synthesis, 37RC cells uninfected or

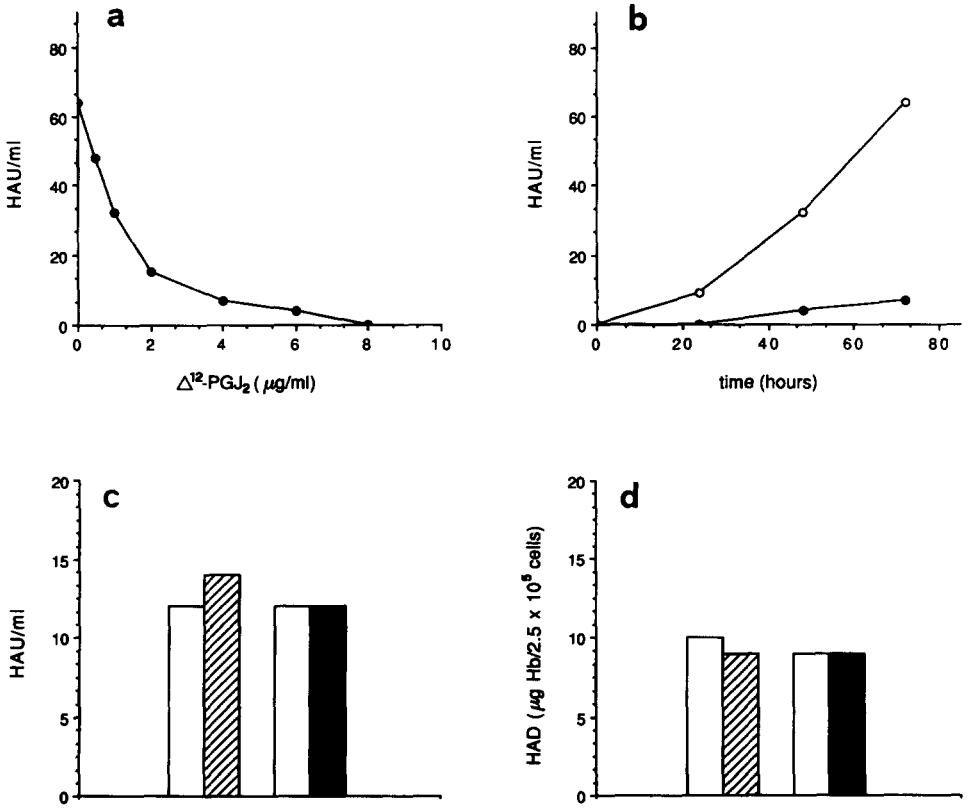

Fig. 1. Effect of Δ^{12}-PGJ_2 on SV production. (a) Dose–response inhibition of SV production. (b) SV production at different times p.i. in 37RC cells treated with 4 μg/ml Δ^{12}-PGJ_2 (●) or ethanol control (○). (c,d) SV treatment with Δ^{12}-PGJ_2 (4 μg/ml) before (□) or during (■) infection had no effect on virus production, measured in HAU (c) or HAD (d) 24 h p.i.; □ = control. S.E.M. = 0 in c and d indicates that titres were identical for each pair of samples.

infected with SV were treated with Δ^{12}-PGJ_2 (4 μg/ml) or control diluent soon after infection, and labelled with [^{35}S]methionine for the following 24 h in the presence of Δ^{12}-PGJ_2. After determining the radioactivity incorporated into TCA-insoluble material, cell extracts were separated by SDS-PAGE and processed for autoradiography. Fig. 2a shows that Δ^{12}-PGJ_2 treatment induced the synthesis of at least three cellular proteins, the molecular weight of which (110, 90, and 72-kDa respectively) corresponded to the molecular weight of members of the major HSP families (HSP110, HSP90 and HSP70 respectively). Δ^{12}-PGJ_2 also increased the synthesis of a 36-kDa and a 66-kDa protein, while it inhibited the synthesis of a 32-kDa cellular polypeptide. Similar alterations of cellular proteins were found in SV-infected cells treated with Δ^{12}-PGJ_2.

HSPs, or stress proteins, are a specific set of polypeptides, the synthesis of which is induced by heat shock or other environmental stresses; they can be divided into five families with M_rs of 15–30 kDa (low M_r HSP), 60 kDa

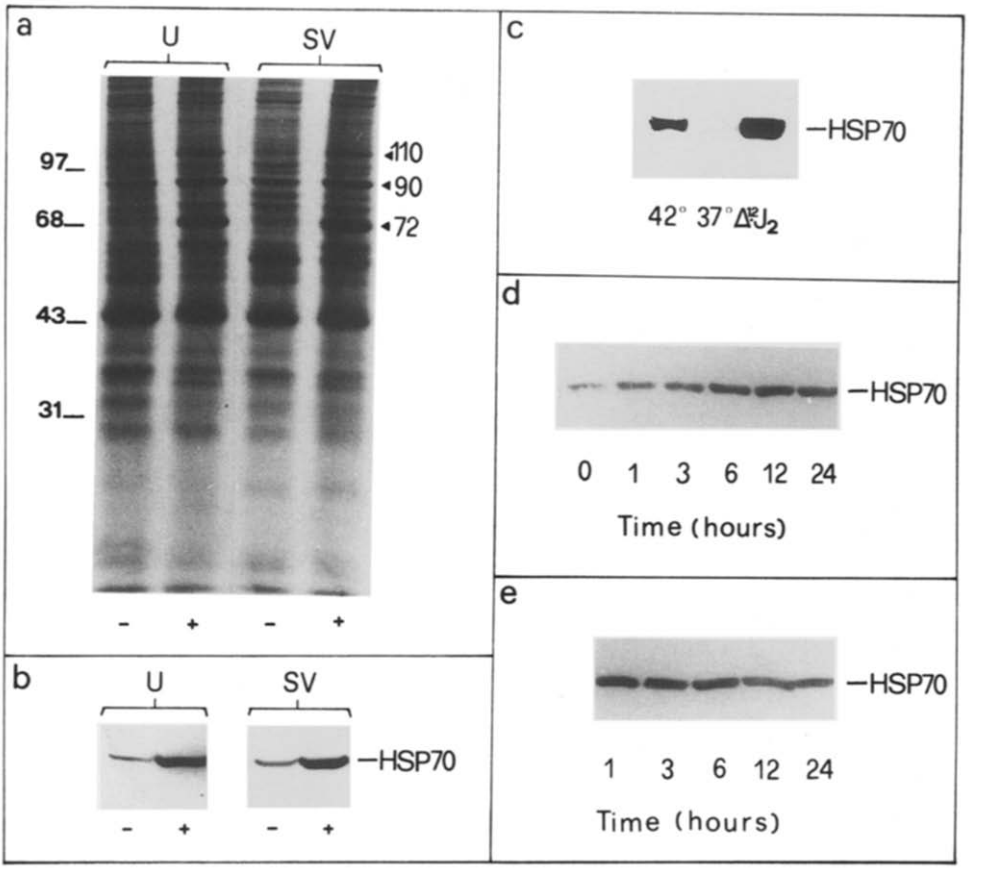

Fig. 2. Induction of HSP70 by Δ^{12}-PGJ_2. (a) SDS-PAGE analysis of [^{35}S]methionine-labeled polypeptides in uninfected (U) or SV-infected (SV) cells treated with Δ^{12}-PGJ_2 (+) or control diluent (−). Densitometric analysis of autoradiographic patterns of uninfected cells after Δ^{12}-PGJ_2 treatment showed a 420%, 180% and 230% increase in the synthesis of 3 polypeptides of 72 kDa, 90 kDa and 110 kDa M_r respectively, as compared to control. Similar results were found in SV-infected cells. (b) Equal amounts of protein from samples treated as in (a) were processed for immunoblot analysis using anti-72/73-kDa HSP MAbs. (c) Comparison of Δ^{12}-PGJ_2-induced HSP70 to HSP70 induced by heat shock. (d) Accumulation of HSP70 at different times after Δ^{12}-PGJ_2 treatment, or (e) at different times after Δ^{12}-PGJ_2 removal.

(HSP60), 70 kDa (HSP70), 90 kDa (HSP90) and 110 kDa (HSP110) (Lindquist and Craig, 1988). In the eukaryotic cell, HSPs are generally present as multigene families with members being expressed in unstressed cells (constitutive HSPs) as well as following heat treatment (inducible HSPs) (Lindquist and Craig, 1988; Schlesinger, 1990). Constitutive HSP70 proteins are needed for the import of several proteins into eukaryotic cell organelles, and can bind to and, in the presence of ATP, dissociate protein complexes. Inducible HSP70s, which are known to bind to partially denatured proteins and to protect cells from the effect of stress, have been also implicated in thermotolerance, regulation of cell growth and differentiation, and viral infection (Schlesinger, 1990).

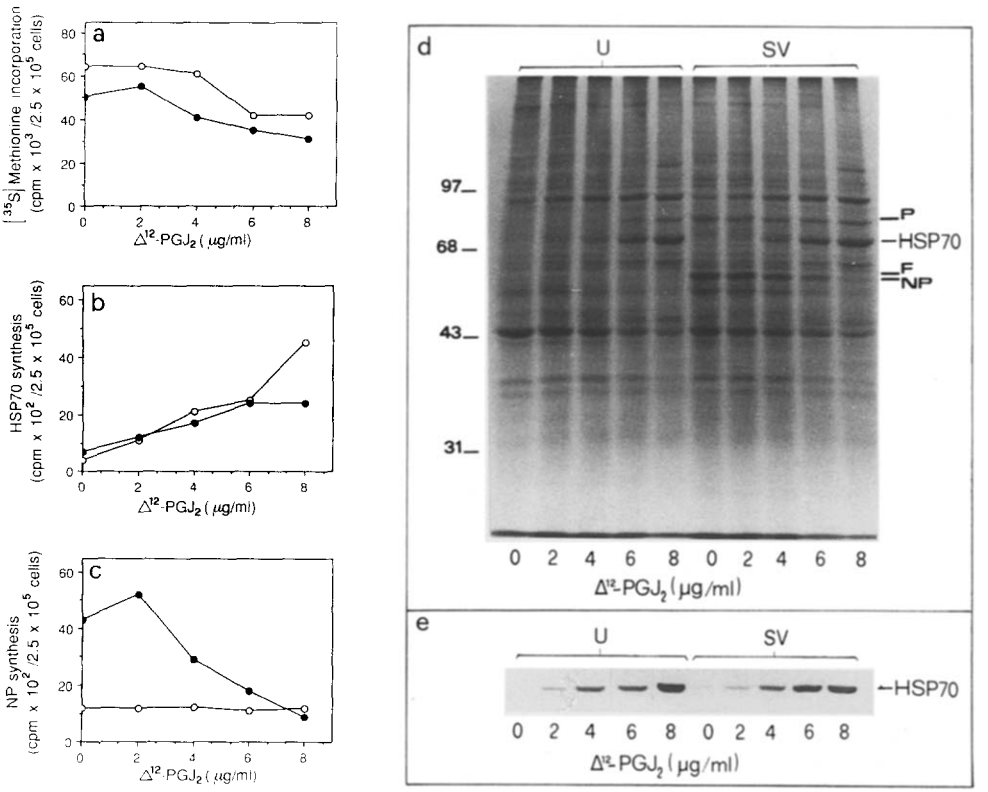

Fig. 3. Dose-dependent effect of Δ^{12}-PGJ_2 on cellular and viral protein synthesis. (a) [^{35}S]methionine incorporation into TCA-insoluble material of uninfected (○) or SV-infected (●) 37RC cells, 24 h p.i. [^{35}S]methionine-labeled proteins were processed for SDS-PAGE analysis and autoradiography (samples containing the same amount of radioactivity were loaded onto gels, d) or for immunoblot analysis (samples containing an equal amount of protein were loaded on gels, e). HSP70 synthesis (b) and virus NP protein synthesis (c) were quantified in uninfected (○) or SV-infected (●) 37RC cells, by cutting out the relevant bands from dried gels, and counting the radioactivity in a β-scintillation counter.

In order to identify the PG-induced proteins as HSPs, confluent monolayers of uninfected or SV-infected 37RC cells were treated with Δ^{12}-PGJ_2 1 h after the infection period. After 24 h, cells were lysed in lysis buffer and, after protein determination, an equal amount of protein from each sample was separated by SDS-PAGE and processed for immunoblot analysis using anti-human 72/73 kDa HSP monoclonal antibodies. Immunoblot analysis revealed the presence of a unique band in both uninfected or SV-infected cells identifying the 72 kDa protein as an HSP70 (Fig. 2b). HSP70 is constitutively synthesized at low levels in 37RC cells and levels of constitutive synthesis vary depending on growth conditions and serum concentration, as reported previously in other cell lines (Morimoto et al., 1990). No apparent difference in the levels of HSP70 was found in SV-infected cells 24 h p.i. as compared to uninfected 37RC cells (Fig. 2a,b). HSP70 synthesis was increased several-fold above constitutive levels after Δ^{12}-PGJ_2-treatment in both uninfected and SV-infected cells (Fig. 2b). To

investigate the extent of Δ^{12}-PGJ_2-induced synthesis as compared to synthesis after heat shock (HS), 37RC cells were either heat-shocked (42°C for 20 min), or kept at 37°C, or kept at 37°C and treated with Δ^{12}-PGJ_2. After 24 h, cell extracts were processed for immunoblot analysis, which revealed that Δ^{12}-PGJ_2 increased HSP70 levels more than HS (Fig. 2c). To examine the kinetics of Δ^{12}-PGJ_2-induced HSP70 synthesis, proteins from 37RC cells were collected at various times after the addition of Δ^{12}-PGJ_2, and processed for immunoblot analysis. Fig. 2d shows that accumulation of HSP70 started from 1–3 h after treatment and continued for the following 24 h. To investigate the stability of the PG-induced HSP70, Δ^{12}-PGJ_2 was removed by repeated washings from the medium of 37RC cells pretreated for 24 h, and fresh medium was added. At different times after removal, samples were collected and processed for immunoblot analysis, which revealed that Δ^{12}-PGJ_2-induced HSP70 is stable for at least 24 h after synthesis (Fig. 2e).

Fig. 3 shows the dose-dependent effect of Δ^{12}-PGJ_2 on cellular and viral protein synthesis, and on HSP70 induction. 37RC cells were infected with SV and treated with different concentrations of Δ^{12}-PGJ_2 soon after infection. Protein synthesis was measured in uninfected or SV-infected cells, by [^{35}S]methionine incorporation (24 h pulse) into TCA-insoluble material (Fig. 3a). Proteins were separated by SDS-PAGE and processed for autoradiography or immunoblotting. HSP70 synthesis was induced by Δ^{12}-PGJ_2 in a dose-dependent fashion (Fig. 3b,d,e). The concentration of 4 μg/ml, which inhibited virus replication by more than 95%, caused a dramatic induction of HSP70 synthesis (Fig. 3b,d,e), without inhibiting protein synthesis (Fig. 3a). SV-infection did not appear to influence the cellular response to Δ^{12}-PGJ_2. At the dose of 2 μg/ml, which inhibited virus replication by more than 80%, Δ^{12}-PGJ_2 did not affect SV-protein synthesis (Fig. 3a,c,d); however, quantitative analysis of autoradiographic patterns revealed that higher doses of Δ^{12}-PGJ_2 caused a dose-dependent decrease in the synthesis of the virus nucleocapsid structural protein NP (Fig. 3c). This protein is known to have a structural function as well as to interact with the auxiliary nucleocapsid proteins (L and P) that carry out viral RNA synthesis (Deshpande and Portner, 1984).

It has been shown that PGA_1 suppresses SV replication by inhibiting virus protein glycosylation (Santoro et al., 1989a). In order to investigate whether Δ^{12}-PGJ_2 could affect SV protein glycosylation or could induce the synthesis of the 78-kDa glucose-regulated stress protein or BIP, which is synthesized in response to defective protein glycosylation (Pelham, 1986), 37RC cells uninfected or infected with SV were treated with PGA_1, Δ^{12}-PGJ_2 (4 μg/ml) or with the glycosylation inhibitor tunicamycin (2 μg/ml) soon after infection. Cells were labeled with either D-[6-^{3}H]glucosamine hydrochloride (20 μCi/10^6 cells, 24-h pulse starting 3 h p.i.) or [^{35}S]methionine (10 μCi/10^6 cells, 2-h pulse starting 24 h p.i.). Fig. 4 shows that: (1) HSP70 was still synthesized at high rate 24 h after Δ^{12}- PGJ_2 treatment, while synthesis had gone back to control levels 24 h after PGA_1 treatment (Fig. 4a); (2) the Δ^{12}-PGJ_2-induced HSP70 migrated differently in SDS/PAGE than the 78-kDa glucose-regulated stress protein,

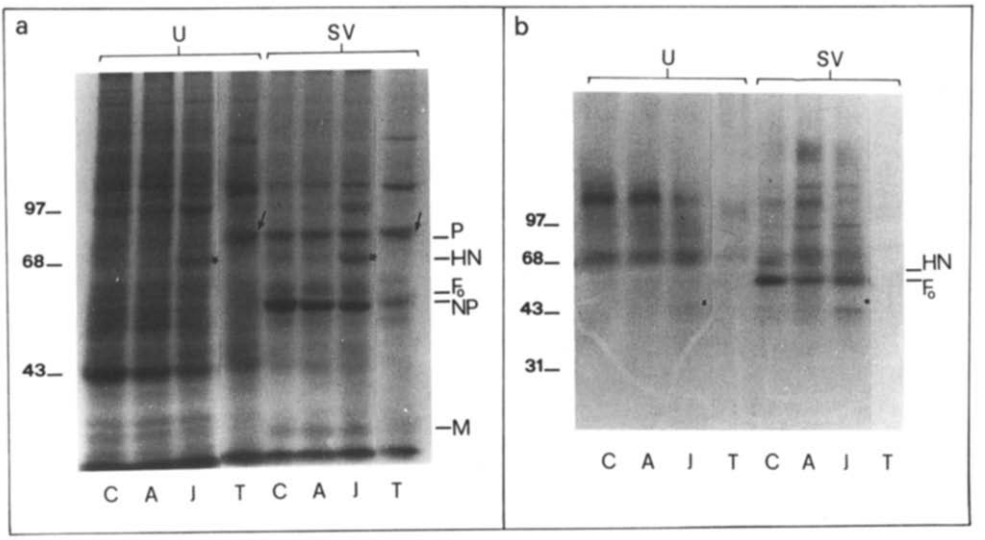

Fig. 4. SDS-PAGE analysis of [^{35}S]methionine-labeled (a) or [^{3}H]glucosamine-labeled (b) proteins after treatment with PGA_1 (A), Δ^{12}-PGJ_2 (J), tunicamycin (T) or diluent control (C) in uninfected (U) or SV-infected (SV) 37RC cells. (a) HSP70 (indicated by asterisk) was still actively synthesized 24 h after Δ^{12}-PGJ_2 treatment, but not after PGA_1 treatment. Arrow indicates the 78-kDa protein induced by tunicamycin. (b) Asterisks indicate the 47-kDa protein glycosylated after Δ^{12}-PGJ_2 treatment.

which was instead induced after tunicamycin treatment (Fig. 4a); (3) Δ^{12}-PGJ_2-treatment caused a marked (approximately 45%) inhibition of glucosamine incorporation into the viral HN and F_o proteins, as well as in a 118-kDa cellular polypeptide (approximately 55%) (Fig. 4b); (4) Δ^{12}-PGJ_2 induced the glycosylation of a 47-kDa cellular protein in both uninfected and SV-infected cells (Fig. 4b).

Δ^{12}-PGJ_2 has been previously shown to inhibit DNA virus (herpes simplex virus) replication in human embryo fibroblasts by inhibiting RNA transcription (Yamamoto et al., 1987). In the present report we show that Δ^{12}-PGJ_2 can suppress the production of mature infectious virus particles in monkey kidney cells infected with SV, an RNA virus belonging to the paramyxovirus group. Even though synthesis of viral RNA and proteins was delayed by several hours by Δ^{12}-PGJ_2 treatment in this model (data not shown), we have found that by 24 h p.i., virus proteins were actively synthesized, while virus production was almost completely inhibited. These data suggest an effect on protein assembly and virus maturation. As previously reported for PGA_1, a decrease in glucosamine incorporation into virus glycoproteins was detected after Δ^{12}-PGJ_2 treatment. These effects were accompanied by the synthesis of HSPs. Δ^{12}-PGJ_2 action differs from the one previously described for PGA_1 in at least three aspects: (1) it induced the synthesis of several HSPs at the conditions in which PGA_1 induced only HSP70 synthesis (Amici and Santoro, 1991); (2) differently from PGA_1, HSP70 was still actively synthesized 24 h after Δ^{12}-PGJ_2 treatment;

(3) Δ^{12}-PGJ_2 also caused the glycosylation of a still unidentified 47-kDa cellular protein.

While it is well established that HSPs induction is a general response of mammalian cells to cyclopentenone PGs (Amici and Santoro, 1991; Santoro et al., 1989b, 1990), it remains to be determined whether HSPs play a role in the antiviral activity of these compounds. The possibility that HSPs could interfere with virus protein synthesis and maturation, which has been previously suggested in heat-shocked cells (Yura et al., 1987), becomes attractive in the view of the induction of HSP synthesis during physiologically relevant increases in body temperature (Cosgrove and Brown, 1983).

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