INTRODUCTION

Medicinal plants or extracts of herbs are extensively screened to identify novel molecules able to prevent virus replication (Mantani et al., 1999; Simoes et al., 1999; Yukawa et al., 1996; Hattori et al., 1995). We have found that crude leaf extracts from members of the Meliaceae family display an antiviral activity against various viruses (Cordoba et al., 1991; Benencia et al., 1997; Andrei et al., 1994). Melia azedarach L. (Meliaceae), also known as the chinaberry tree, is a deciduous tree native to India and has long been recognized for its medicinal and insecticidal properties (Bohnenstengel et al., 1999). Its fruit has been used traditionally for the treatment of a variety of diseases, especially dermatitis and rubella (Kim et al., 1999). We have demonstrated that leaf extracts from M. azedarach L. inhibit the multiplication of viruses such as herpesvirus, Junin virus, Sindbis virus, vesicular stomatitis virus (VSV), poliovirus, pseudorabies virus and Tacaribe virus in vitro with no cytotoxic effects (Wachsman et al., 1982, 1987; Descalzo & Coto, 1989). The antiviral activity has been ascribed to a partially purified inhibitor designated meliacine (MA) and its mode of action has been investigated in several virus–cell systems (Wachsman et al., 1998; Castilla et al., 1998; Alche et al., 2002). MA is able to trigger an antiviral state in a variety of continuous and primary cell cultures when added before infection, and it has been shown that MA induces a refractory state to VSV infection within 2 h of cell pretreatment, which extends for over 12 h after its removal (Andrei et al., 1988).

Many compounds of plant origin exhibiting antiviral activity have been described. These include alkaloids (Martin, 1987), flavonoids (Lin et al., 1999), terpenes and poly saccharides (Bourne et al., 1999), lignans (Charlton, 1998), steroidal glycosides (Ikeda et al., 2000) and proteins (Aoki et al., 1995). Recently, we reported the isolation and purification of 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM) from leaf extracts of M. azedarach L., a tetranortriterpenoid with in vitro insecticidal properties (Lee et al., 1991). We established that CDM inhibits VSV and herpes simplex virus type 1 (HSV-1) multiplication in vitro when added after infection, with low cytotoxicity (Alché et al., 2003).

Since CDM exhibits antiviral activity against VSV and HSV-1, we hypothesized that it could be the molecule responsible for the broad spectrum of MA antiviral action previously shown. Therefore, the aim of the present paper was to

Block of vesicular stomatitis virus endocytic and exocytic pathways by 1-cinnamoyl-3,11-dihydroxymeliacarpin, a tetranortriterpenoid of natural origin

Andrea A. Barquero, Laura E. Alché and Celia E. Coto

Laboratory of Virology, Department of Biochemistry, School of Science, University of Buenos Aires, Pabellón II, Piso 4to, Ciudad Universitaria, C1428BGA Buenos Aires, Argentina

Previously, it has been shown that 1-cinnamoyl-3,11-dihydroxymeliacarpin (CDM), a natural compound isolated from leaf extracts of Melia azedarach L., inhibits the vesicular stomatitis virus (VSV) multiplication cycle when added before or after infection. Here, we have established that the lack of VSV protein synthesis in CDM pre-treated Vero cells is ascribed to the inhibition of an initial step during virus multiplication, although indirect immunofluorescence (IFI) studies confirmed that the binding and uptake of [35S]methionine-labelled VSV was not affected by CDM pre-treatment. Instead, our findings revealed that this compound impedes the uncoating of VSV nucleocapsids in pre-treated Vero cells, since the antiviral action of CDM was partially reversed by inducing VSV direct fusion at the plasma membrane, and VSV M protein fluorescence was confined to the endosomes, even 2 h post-internalization. Furthermore, CDM induced cytoplasmic alkalinization, as shown by acridine orange staining, consistent with the inhibition of virus uncoating. Although VSV proteins are synthesized when CDM is added after infection, IFI studies revealed that G protein was absent from the surface of infected cells and co-localized with a Golgi marker. Therefore, CDM inhibits the transport of G protein to the plasma membrane.

Taken together, these findings indicate that CDM exerts its antiviral action on the endocytic and exocytic pathways of VSV by pre- or post-treatment, respectively.
unravel both the mechanism of action of CDM on the VSV multiplication cycle and to investigate its eventual effect on the induction of a refractory antiviral state.

**METHODS**

**Cells and virus.** Vero cells were grown in Eagle’s minimal essential medium supplemented with 5% inactivated calf serum (MEM 5%) and 50 µg gentamicin ml−1 and maintained after monolayer formation in MEM supplemented with 1.5% inactivated calf serum (MEM 1.5%). The Indiana strain of VSV was plaque-purified and propagated on Vero cell monolayers.

**Antiviral compound.** CDM was purified from the leaves of *M. azedarach* L., as described by Alché *et al.* (2003), solubilized in MEM 1.5% to a final concentration of 1 mg ml−1 and stored at −20°C.

**Acridine orange staining of living cells.** Vero cells grown on coverslips were stained with acridine orange (1 µg ml−1) for 15 min at 37°C, washed twice with cold PBS, mounted in, and visualized on a Zeiss Axioplan fluorescence microscope (magnification ×400).

**Analysis of VSV proteins by SDS-PAGE.** Vero cells were grown in 24-well plates and infected with VSV, then incubated in MEM 1.5% until 5 h post-infection (p.i.). Protein labelling was carried out using 10 µCi [35S]methionine ml−1 (1175 Ci mmol−1; New England Nuclear) added from 5 to 7 h p.i. in methionine-free medium. The radiolabelled monolayers were dissolved in Laemmli buffer (Laemmli, 1970) and aliquots of each sample were heated at 90°C for 5 min and analysed by 10% SDS-PAGE.

**Preparation of [35S]methionine-labelled VSV.** Vero cells cultured in 750 cm² flasks were infected with VSV at an m.o.i. of 0.01 p.f.u. per cell. At 15 h p.i., the maintenance medium was replaced with methionine-free medium containing 10 µCi [35S]methionine ml−1 (1175 Ci mmol−1; New England Nuclear). After an 8–10 h incubation period, the medium was collected and cellular debris was removed. The virus was concentrated by centrifugation at 26,000 r.p.m. for 2 h at 4°C in a Beckman SW 28 rotor. Further purification was achieved by centrifugation at 46,000 r.p.m. for 2 h at 4°C in a Beckman SW 55 Ti rotor in a sucrose discontinuous gradient. After centrifugation, the virus band was collected, pelleted and titrated. The virus titre obtained was 4 × 10⁸ p.f.u. ml−1, corresponding to 1500 p.f.u. cm²⁻¹.

**Binding and uptake assays.** To measure binding, 1.4 × 10⁴ and 3 × 10⁴ c.p.m. of [35S]labelled VSV diluted in binding medium (serum-free MEM containing 0.5 mg BSA ml⁻¹ and 3 mM HEPES) were allowed to bind to Vero cells at 0°C and 37°C, respectively, with gentle agitation. At indicated times, the total cell-associated radioactivity was measured by lysing the cells with 0.1 M NaOH/1% SDS and directly adding a detergent-based scintillation fluid. The internalized radioactivity was counted after treating the cells with 1 mg proteinase K ml⁻1 to remove surface-associated virus.

**Endocytosis and degradation were studied by binding 3 × 10⁴ c.p.m. [35S]labelled VSV to cells at 0°C and then raising the temperature to 37°C.** At different times p.i., the internalized radioactivity was counted as described above. The appearance of TCA-soluble [35S]methionine in the medium was determined by precipitation with an equal volume of 10% TCA on ice for 45 min and centrifugation for 5 min at 10,000 g. The supernatant was then counted for radioactivity. In all cases, radioactivity was counted in a liquid scintillation counter (240 CL/D Packard).

**Indirect immunofluorescence assay (IFA).** Vero cells grown on coverslips to 70% confluence were infected with VSV. For surface and internal staining, Vero cells were fixed with methanol for 10 min at −20°C. Fixed cells were then incubated for 30 min at 37°C with rabbit anti-G protein polyclonal antibodies or mouse anti-M protein monoclonal antibodies (mAbs) (kindly provided by Pablo Grigera, CEVAN, Buenos Aires, Argentina). Cells were then incubated with goat anti-rabbit or anti-mouse IgG secondary antibodies, respectively, conjugated to FITC or TRITC (Sigma) for 30 min at 37°C. For surface IFA staining, the addition of primary antibodies to VSV-infected cells for 30 min at 4°C was done prior to fixation with methanol. In both cases, coverslips were rinsed and mounted. Cells were photographed with a Zeiss microscope with epifluorescence optics.

**Confocal microscopy.** Vero cells grown on coverslips in a 24-well plate were transfected with a cDNA encoding galactosyltransferase T2 fused with the enhanced green fluorescent protein (GaT2–GFP) (Giraudo *et al.*, 2001), provided by Hugo Macchioni. Lipofectin reagent (Gibco) was used for transfection of Vero cells with 2 µg of plasmid DNA per well. After 24 h transfection, cells were infected with VSV at an m.o.i. of 1 p.f.u. per cell. At 6 h p.i., cells were processed for G protein staining using an anti-rabbit TRITC-conjugated antibody. Coverslips were mounted and analysed with an Olympus FB300 confocal microscope. Images were collected and processed using Fluoview version 3.2 and Adobe Photoshop software.

**RESULTS**

**Correlation of the effect of CDM on VSV growth and alkalinization of Vero cell vacuolar compartments**

In previous reports it was shown that Vero cell pretreatment with partially purified *M. azedarach* L. leaf extracts induces an antiviral state as well as an increase in the pH of intracellular acidic compartments in BHK cells (Andreï *et al.*, 1988; Wachtsman *et al.*, 1998). Hence, we decided to investigate whether CDM was the molecule responsible for both effects.

Initially, we established that CDM prevented VSV multiplication in Vero cells when added 2 h before infection, exhibiting 50% inhibition of virus yield at a concentration of 0.75 µM and 50% cytoxicity at a concentration above 520 µM (data not shown).

The effect of CDM on both virus multiplication and endosomal pH was examined in Vero cell monolayers treated with 7.5 µM CDM for 2 h at 37°C and infected with 200 p.f.u. VSV per well at 0, 2, 4, 8, 10 and 24 h post-treatment (p.t.). A control set of uninfected cells grown on coverslips was stained with acridine orange at the same time intervals p.t. When Vero cells were infected immediately after treatment or as long as 10 h p.t., there was more than 90% inhibition in a plaque reduction assay. In contrast, susceptibility of cells to virus infection was restored by 24 h p.t. (Fig. 1A).

The vital fluorescence microscopic study showed that untreated cells exhibited a bright orange punctuate fluorescence concentrated in low-pH vesicles generally with perinuclear distribution (Fig. 1B). However, the pH of acidic intracellular vesicles from CDM-treated cells was...
**Fig. 1.** Effect of CDM on VSV multiplication and endosomal pH. Vero cells treated with CDM for 2 h at 37 °C (black bars) or not (white bars) were infected with 200 p.f.u. VSV at various times post-treatment. The number of p.f.u. was counted at 48 h post-treatment (A). CDM-treated uninfected cells were stained with acridine orange at 0 (C), 2 (D), 4 (E), 8 (F), 10 (G) or 24 (H) h post-treatment. (B) Untreated Vero cells. Magnification × 400.

CDM affects VSV endo- and exocytic pathways.
markedly affected to such a level that only faint granular fluorescence was observed until 4 h after drug removal (Fig. 1C–E). The inhibitory effect of CDM was still evident at 10 h p.t., since the amount and intensity of granular orange fluorescence in CDM-treated cells were significantly lower than that of untreated cells (Fig. 1F and G). At 24 h p.t., the acidic luminal pH of vesicles was not completely restored, although it was much more like the corresponding untreated cells (Fig. 1H).

In conclusion, CDM induces a refractory state in Vero cell monolayers that is maintained for at least 10 h p.t., as well as causing the pH of intracellular vesicles to become alkaline, as is also the case with MA.

Effect of CDM on VSV protein expression

We have previously demonstrated that CDM inhibits VSV multiplication when added after infection in a multicyle growth experiment (Alché et al., 2003). A CDM dose-dependent inhibitory effect was also observed under single-step growth conditions. Vero cells infected with VSV (m.o.i. = 1) for 1 h at 37°C were treated with different concentrations of CDM and at 6 h p.i., supernatants from treated and untreated cultures were titrated in a plaque assay. VSV titres after treatment with 0, 75, 1, 5, 7.5, 15 and 75 μM CDM were $10^6$, $10^5$, $10^5$, $10^5$, $10^5$, $10^5$, and $10^5$ p.f.u. ml$^{-1}$, respectively, whereas an infectivity of $1.5 \times 10^9$ p.f.u. ml$^{-1}$ was obtained in infected but untreated cells. Virus replication was also suppressed by 90 and 91% when 75 μM CDM was added to the cultures for 0–3 and 3–6 h p.i., respectively.

In view of these results, we decided to investigate whether the antiviral activity of CDM targets unique or different targets in the VSV multiplication cycle by analysing the expression of viral proteins.

Vero cells were treated with different concentrations of CDM before or after infection with VSV at an m.o.i. of 1 p.f.u. per cell. At 5 h p.i., cells were pulse-labelled with $[^{35}S]$methionine for 2 h and the labelled polypeptides were analysed by SDS-PAGE. At this time post-infection, host translation has been shut-down and infected cells mostly synthesize VSV proteins. When added before infection, CDM decreased the levels of VSV proteins in a dose-dependent manner and restored the expression of cellular proteins (Fig. 2A). The inhibition observed suggested that CDM blocks the synthesis of viral proteins or an initial event in VSV infection in pre-treated cells.

The addition of CDM after infection appeared not to suppress VSV protein synthesis (Fig. 2B). Thus, a later step in the VSV infectious cycle must also be inhibited by CDM, since a reduction in virus yield was observed.

Effect of CDM on the early steps of VSV multiplication

To assess the effect of CDM pre-treatment on the initial stages of the infection cycle, radioactively labelled VSV was used to determine the interaction of VSV and cells at 0°C and 37°C. Vero cells were treated with 75 μM CDM for 2 h at 37°C, then infected with $[^{35}S]$VSV at 0°C. Less than 10% of the added virus became cell-associated after 1 h at 0°C, indicating that CDM pre-treatment did not interfere with VSV binding at 0°C (data not shown).

To determine the effect of CDM on virus internalization, the amount of both total cell-associated and proteinase K-resistant radioactivity was measured. A partial inhibition (44%) of total cell-associated VSV in pre-treated cells was observed (Table 1). However, the internalized radioactivity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total c.p.m. (mean ± SD)</th>
<th>Internalized c.p.m. (mean ± SD)</th>
<th>Internalization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$5816 \pm 524$</td>
<td>$1483 \pm 162$</td>
<td>25.5</td>
</tr>
<tr>
<td>CDM</td>
<td>$3273 \pm 329$</td>
<td>$1630 \pm 176$</td>
<td>49.8</td>
</tr>
</tbody>
</table>

Fig. 2. Synthesis of VSV proteins in CDM-treated cells. Vero cells infected with VSV were treated with different concentrations of CDM before (A) or after (B) adsorption. Cells were pulse-labelled with $[^{35}S]$methionine from 5 to 7 h p.i. and analysed as described in Methods. CC, Mock-infected cells; CV, infected untreated cells. VSV proteins are indicated on the left.
did not differ significantly from that obtained in the untreated culture (Table 1). These experiments indicated that CDM diminishes the amount of surface-associated VSV at 37°C.

Next, the rate of 35S-labelled VSV endocytosis and degradation was studied. As seen in Fig. 3, about 1% of the added VSV entered the cells within 5 min upon warming and this uptake did not increase with time. The amount of acid-soluble radioactivity began to appear in the medium within 40 min of warming. In cells pre-treated with CDM, the endocytosis of VSV was not affected, but virus degradation into TCA-soluble material was significantly inhibited at 60 min post-adsorption in pre-treated compared with untreated cells (P = 0.05, Student’s t-test) (Fig. 3). These results suggest that the viral stage blocked in CDM pre-treated cells follows the binding and internalization steps.

To support the above findings, an IFI staining using anti-M protein mAb was performed. After virus adsorption, one set of cells on coverslips was fixed directly (T0), while the other was warmed at 37°C for 1 h before fixation (T1). At T0, the surface of infected cells was covered with bright dots of different sizes (Fig. 4A), whereas at T1, most of the surface fluorescence had disappeared and internal M protein was distributed in a punctuate pattern (Fig. 4C and E). Similar results were obtained in CDM pre-treated cells (Fig. 4B, D and F), indicating that the antiviral compound did not affect the binding and uptake of VSV.

Thus, we predicted that CDM pre-treatment should affect the next step in the cycle of VSV: the release of viral nucleocapsids into the cytoplasm.

**Effect of CDM on low-pH-induced VSV fusion entry**

Since the uncoating of VSV depends on a membrane fusion event and VSV can be made to fuse directly with the plasma membrane by lowering the pH of the medium, we decided to examine the effect of CDM on the acid-induced virus fusion activity. For this purpose, Vero cells infected with VSV at an m.o.i. of 10 p.f.u. per cell for 1 h at 0°C were incubated with MEM pH 5 for 5 min at 37°C. The medium was then replaced with MEM pH 7-4 and virus yield was measured at 20 h p.i. No difference in virus titre corresponding to infected cells treated either with MEM pH 5 or MEM pH 7-4 was observed. However, whereas CDM pre-treatment reduced VSV production by 3 logs in cells maintained in MEM pH 7-4, only a 1 log reduction in VSV yield was found in cells briefly exposed to MEM pH 5 (Fig. 5A, i).

Thus, we speculated that a decrease in the extracellular pH could be affecting the intracellular compartment acidification due to CDM instead of inducing virus fusion. When the culture medium was adjusted to either pH 7-4 or 5 immediately after CDM pre-treatment and before VSV infection, no reversion of the refractory antiviral state of treated Vero cells was observed. As expected, CDM pre-treatment reduced virus yield by 3 logs compared with the untreated control, regardless of the pH of the culture medium (Fig. 5A, ii). Acridine orange staining of Vero cells pre-treated with CDM demonstrated that low-pH treatment did not decrease the alkaline pH induced by CDM (Fig. 5B).

Additional data supporting the impairment of the VSV endocytic pathway by CDM were provided by morphological analysis of the M protein, which was expressed in untreated Vero cells at 2 h p.i. independently of the pH of the medium (Fig. 5C, i and ii). However, in CDM pre-treated infected cultures, M protein was only synthesized when VSV fusion was induced at pH 5 (Fig. 5C, iv), whereas a restricted and punctuate fluorescence pattern was observed at pH 7-4 (Fig. 5C, iii).

Therefore, VSV inhibition due to CDM pre-treatment can be by-passed by inducing virus fusion at the cell surface.

**Intracellular localization of M and G VSV proteins in CDM post-treated cells**

Since VSV proteins are synthesized in the presence of CDM when it is added after infection (Fig. 2B), we decided to visualize the intracellular localization of M and G proteins in infected and treated cells by total or surface IFI staining.

No difference in M protein intracellular localization in infected control cells and CDM post-treated infected cells were observed (Fig. 6B and C, respectively). With respect to G protein cytoplasmic expression, no significant difference between the number of fluorescent cells from untreated and CDM-treated cultures was detected (Fig. 6E and F).
Strikingly, whereas the G protein was distributed throughout the cytoplasm and the plasma membrane of untreated infected cells (Fig. 6E), it appeared to be associated with the perinuclear region in the majority of cells treated with CDM (Fig. 6F). The antiviral treatment caused an inhibition of 67% in the number of fluorescent cells expressing
Fig. 5. Low pH-induced VSV fusion entry in CDM pre-treated cells. (A) VSV production in CDM pre-treated cells (75 μM for 2 h at 37 °C) incubated with MEM pH 7.4 (black bars) or MEM pH 5 (white bars) before (i) and after (ii) virus adsorption. Virus titres were determined by a plaque assay on Vero cells. Data are means ± SD of two experiments. (B) Acridine orange staining of Vero cells treated with 75 μM CDM for 2 h at 37 °C and briefly exposed to MEM pH 7.4 (i) or MEM pH 5 (ii). Magnification × 400. (C) Vero cells treated with 75 μM CDM (iii, iv) or not (i, ii) for 2 h at 37 °C were infected with VSV (m.o.i. = 1000) for 1 h at 0 °C. After adsorption, cells were washed with PBS and incubated with MEM pH 5 (ii, iv) or MEM pH 7.4 (i, iii) for 5 min at 37 °C. Both media were then replaced with MEM pH 7.4 and cells were incubated at 37 °C. After 2 h p.i., cells were fixed and an IIF assay with an anti-M mAb was carried out. Magnification × 1000.
G protein at their surface (Fig. 6I). These results demonstrated that the intracellular transport of the VSV G protein is affected by CDM.

To determine whether the marked accumulation of G protein observed in the perinuclear region of CDM-treated cells localized to the Golgi apparatus, cells were transfected with a cDNA encoding the GalT2–GFP fusion protein as a Golgi marker. By performing IFI staining, we found that VSV G protein fluorescence co-localized with GalT2–GFP in CDM-treated cells, suggesting that the VSV G protein is associated with the Golgi complex (Fig. 7).

DISCUSSION

The attempt to elucidate the target for the anti-VSV activity of CDM led us to deal with two phenomena: the ability of CDM to induce a refractory state in Vero cells by pre-treatment and the direct antiviral activity of CDM achieved when the compound is supplied after infection.

CDM was shown to induce cytoplasmic alkalinization. We strongly suggest that the establishment of the refractory state is associated with this alteration, since the anti-VSV activity correlated closely with the increase in pH for as long as 10 h (Fig. 1).

Since the acidification of intracellular endosomes is required for virus endocytosis, the inhibition of entry of VSV nucleocapsids into Vero cells by the endocytic pathway was expected. In this sense, the first clue for the involvement of an early step in the VSV multiplication cycle affected by CDM was the lack of viral protein synthesis in pre-treated infected cells (Fig. 2A). Further results discounted adsorption as being affected by CDM, although virus internalization was partially inhibited (Fig. 3). This partial inhibition

![Fig. 6. Intracellular distribution of M and G proteins in CDM-treated cells. Vero cells were infected with VSV (m.o.i. = 1) (B, C, E, F, H, I) or not (A, D, G). After 1 h adsorption at 37 °C, cells were treated with 75 μM CDM (C, F, I). At 6 h p.i., IFI staining was performed by adding anti-M mAb to fixed cells (A, B, C) and anti-G polyclonal antibodies prior to (G, H, I) or after (D, E, F) fixation with methanol. Magnification × 1000.](http://vir.sgmjournals.org)

![Fig. 7. Co-localization of G protein with a Golgi marker in CDM-treated cells. Vero cells were transfected with a plasmid containing the GalT2–GFP cDNA (green, B) and 24 h post-transfection, cells were infected with VSV at an m.o.i. of 1 p.f.u. per cell. At 6 h p.i., G protein was detected by using an anti-rabbit TRITC secondary antibody (red, A). Cells were analysed by confocal microscopy; co-localization of G protein and GalT2–GFP appears yellow (C). Magnification × 1000.](http://vir.sgmjournals.org)
did not account for the lack of viral protein synthesis observed and perhaps could be a consequence of an eventual inhibitory effect of CDM on membrane and/or receptor recycling. Similar results were reported by Pérez & Carrasco (1994) in BHK-21 cells infected with Semliki forest virus and treated with bafilomycin A1, which interferes with virus uncoating.

The antiviral effect of CDM was partially reversed when VSV entered cells by direct fusion at the plasma membrane, revealing that VSV was able to enter a multiplication cycle, despite the refractory antiviral state of the cells. At pH 5.7, only 10% of infecting virus directly fuses with cellular membrane (Puri et al., 1988), which would explain the partial reversion of the inhibitory effect of CDM.

It is well known that M protein becomes soluble in the cytoplasm after virus internalization (Rigaut et al., 1991). Since we found a pattern of punctuate fluorescence in CDM pre-treated cells (Fig. 5C, iii), we inferred that M protein was confined to the endosomes due to the inability of virus particles to uncoat, even 2 h post-infection.

Taken together, these findings suggest that delivery of VSV nucleocapsids into the cytoplasm was hampered in CDM pre-treated cells, though this is not enough to explain the anti-VSV activity of CDM when added after infection in a single-cycle growth experiment. The fact that VSV protein synthesis was not affected (Fig. 2B) suggests that a late step in the virus multiplication cycle is hindered by CDM post-treatment.

IFI staining results clearly showed that the G protein did not appear at the surface of CDM-treated infected cells (Fig. 6I) and the cytoplasmic fluorescent pattern observed correlated that G protein accumulated somewhere along the exocytic route (Fig. 6F), even when CDM was added from 3 to 6 h p.i. (data not shown). Confocal microscopy revealed that glycoprotein G was confined to the Golgi complex after CDM treatment (Fig. 7). Hence, CDM appears to be affecting the endocytic and exocytic pathways of VSV as a consequence of its action on the pH of intracellular organelles.

Acidification of vacuolar compartments plays an important role in a variety of cellular processes. Perturbation of the acidic pH of various intracellular organelles by acidicotropic agents, such as weak bases and ammonium chloride, ionophores such as monensin and specific inhibitors of vacuolar (V-) ATPase such as bafilomycin, affects both viral endocytic and exocytic pathways (Sidhu et al., 1999). Although the precise mechanism by which CDM exerts its effects is still unresolved, it is the first tetranortriterpenoid with antiviral activity described responsible for the alkalization of intracellular compartments.

Palokangas et al. (1994) have shown that bafilomycin A1 blocks VSV glycoprotein transport in BHK-21 cells and suggested that normally the Golgi complex is acidified by a vacuolar-type H+-ATPase. Likewise, the inhibition of transport of the VSV G protein by IFN-β may be related to the inhibition of V-ATPase-mediated acidification of the trans-Golgi network (Sidhu et al., 1999). An eventual relationship between the CDM pleiotropic effect and V-ATPase should be investigated to elucidate further whether the antiviral activity observed is a consequence of CDM modulation of V-ATPase activity.

ACKNOWLEDGEMENTS

We are grateful to Dr Alfredo Cáceres (INIMEC-CONICET, Córdoba, Argentina) for his helpful suggestions. The authors wish to thank to Isabel Paz and Guillermo Assad for their technical assistance. This work was supported by a grant from the University of Buenos Aires UBA X-051.

REFERENCES


