The effect of cicloxolone sodium on the replication of vesicular stomatitis virus in BSC-1 cells

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The effect of cicloxolone sodium (CCX) on the replication of vesicular stomatitis virus (VSV) was investigated. The drug was active during all stages of the virus replication cycle, indicating that it does not operate by the specific inhibition of any single essential virus gene product. The drug reduced the number of VSV particles assembled and released by 100- to 1000-fold. Infectious virus yield was reduced 1000- to 10000-fold, giving a 10-fold or greater increase in the particle/p.f.u. ratio. The reduced number of virus particles produced in the presence of CCX results from two superimposed effects: suppression of VSV secondary transcription and viral protein synthesis, and perturbation of virion assembly. The inhibition of VSV assembly is due to impairment of a Golgi apparatus function related to transport of VSV glycoprotein G to the cell surface, and is characterized by accumulation of viral G and M proteins within the cell. Incubation of VSV-infected cells in the presence of two glycosylation inhibitors, tunicamycin and monensin, similarly leads to intracellular accumulation of G and M proteins, suggesting a common mechanism of action affecting VSV virion assembly. The differential effect of CCX concentration on intracellular levels of the L, N and NS proteins was analysed. CCX also possesses a virucidal effect on mature infectious VSV particles in suspension, 300 µM reducing the VSV titre about 10-fold in 24 h at 4 °C or 37 °C. The mode of antiviral activity against VSV is compared with that against herpes simplex virus.

Introduction

Double-blind placebo-controlled clinical trials have indicated that topical treatment of herpes simplex virus (HSV) lesions in man with cicloxolone sodium (CCX) or carbenoxolone (CBX) markedly reduce both healing time and pain associated with lesions (Poswillo & Roberts, 1981; Csonka & Tyrrell, 1984). The anti-HSV activity of the drugs in vitro has been shown to be complex: quantitatively the synthesis, processing and transport of several viral gene products are affected so that the mode of action cannot be related specifically to a single viral protein. The anti-HSV effect is manifest principally at the level of particle assembly and maturation. Grown in the presence of these drugs, HSV particle yields are not only reduced, but more importantly exhibit a dramatically increased particle/p.f.u. ratio, indicating a pronounced loss of virus quality (as defined by relative infectivity) (Dargan & Subak-Sharpe, 1985, 1986a, b; Dargan et al., 1988). In addition, a specific HSV genetic component, identified by a difference in sensitivity between HSV-1 and HSV-2, has been pinpointed to the locations of glycoproteins gH and gC by analysis of intertypic recombinants (Dargan & Subak-Sharpe, 1991). The complex nature of the general antiviral activity against HSV suggested to us that the replication of viruses of unrelated groups might be similarly inhibited by the triterpenoid drugs. We were able to demonstrate that CCX does indeed possess broad range antiviral activity (Galt et al., 1990) and the total yield of viruses from several different families is adversely affected by the presence of CCX in the growth medium; in contrast, a togavirus is not affected.

Galt et al. (1990) assigned viruses to three CCX sensitivity classes (CCX<sup>s</sup>) based on the kinetics of the dose-response curves obtained. The first (CCX<sup>s</sup>-1) (including HSV-1, HSV-2, equine herpesvirus type 1, bovine herpesvirus type 1, influenza A virus and vesicular stomatitis virus (VSV)) exhibits a progressive dose-dependent reduction in infectious virus yield. The second (CCX<sup>s</sup>-2) (including Bunyamwera and Germiston bunyavirus; poliovirus type 1; reovirus type 3 and adenovirus type 5) showed biphasic kinetics, the dose-response curve reaching a plateau of maximum antiviral effect. The third class (CCX<sup>s</sup>-3) [containing Semliki Forest virus (SFV)] appeared to be unaffected by the presence of CCX and at present contains only a togavirus.

Dargan et al. (1992) have found that CCX impairs the release of infectious SFV (CCX<sup>s</sup>-3) from cells and have
detailed the processes affected by CCX antiviral activity for four CCX-2 class viruses. The present study investigates the antiviral activity of CCX against the negative-strand RNA virus VSV (CCX-1) and compares the mechanism of antiviral action against VSV with that against HSV (CCX-1).

**Methods**

**Cells.** BSC-1 cells were used throughout. Cells were grown in Eagle's MEM supplemented with 10% foetal calf serum (EFCo) but when treated with CCX the serum was reduced to 2% (EFC2). BSC-1 cells have been investigated previously by Galt et al. (1990) and found to show little cytotoxic effect at even 300 μM: IC50 > 300 μM after 48 h exposure.

**Drugs.** CCX (Biorex Laboratories) was prepared as described (Dargan & Subak-Sharpe, 1985). Monensin (Mon) (Sigma) was prepared as a 10 mM stock solution in ethanol; tunicamycin (Tun) (Sigma) and actinomycin D (Act D) (Merck, Sharp and Dohme) were prepared as 1 mg/ml stocks in sterile distilled water.

**Virus.** VSV (Indiana serotype) was grown on BSC-1 cell layers infected at a multiplicity of 0-01 p.f.u./cell. After a 2 day incubation at 31 °C the VSV released into the growth medium was harvested and the yield titrated by plaque assay. CCX was purified by exactly the same method as for HSV by Szilagyi & Cunningham (1991). Labelled virus was prepared by infecting BSC-1 cells with 10 p.f.u./cell, then adding 25 μCi/ml [35S]methionine to the culture medium, with or without 150 μM-CCX, and incubating from 1 to 24 h at 37 °C. Virus particles were counted by negative staining in a Siemens 101 electron microscope using phosphotungstic acid, as described by Wildy et al. (1960).

**VSV CCX dose-response.** Confluent monolayers of cells (2 x 10⁶ cells) were infected with VSV at 5 p.f.u./cell and allowed to absorb for 1 h at 37 °C. The cell layer, after three washes with PBS supplemented with 5% calf serum (PBS C5), was then overlaid with 2 ml EFC2 containing various concentrations of drug and incubated at 37 °C for 24 h. To determine total infectious yield the cells were scrapped into the clarified supernatant medium provided the CR virus preparations, whereas the VSV liberated by sonicating the infected cell pellet (made to 2 ml with fresh EFC2) provided the CA virus preparation. The infectious progeny yield was estimated by plaque assay. To monitor any cytotoxic effect, uninfected cells were treated in parallel with CCX and cell viability was determined after 24 h (Dargan & Subak-Sharpe, 1985; Galt et al., 1990).

**One-step VSV growth curve.** Confluent monolayers of 2 x 10⁶ cells were infected with VSV at 5 p.f.u./cell; after the 1 h absorption and washing procedures they were overlaid with EFC2 with or without 300 μM-CCX. At various times post-infection (p.i.) the cultures were harvested, sonicated and the infectious yield was titrated.

**Radiolabelling of cellular and VSV-specified polypeptides.** Confluent monolayers in Linbro trays (5 x 10⁶ cells/well) were infected with 5 or 50 p.f.u./cell. After the absorption and washing procedures the cultures were overlaid with EFC2 (or EFC2 having one-fifth the normal concentration of methionine (Emet-FC2)) containing the indicated concentrations of CCX, Mon or Tun. [35S]Methionine (10 μCi/ml; 900 Ci/mmol) or 10 μCi/ml [14C]glucosamine hydrochloride (50 to 60 mCi/mmol) were added to the mock-infected (m.i.) or VSV-infected cultures in the presence or absence of various concentrations of the drugs.

**SDS-PAGE.** Extraction of protein for SDS-PAGE and the conditions for electrophoresis were as described by Marsden et al. (1976), except that 10% acrylamide gels were used.

**Radiolabelling of RNA.** BSC-1 cells (1 x 10⁶) seeded onto 90 mm tissue culture dishes in EFCo, lacking phosphate were incubated at 37 °C overnight. From 2 h prior to infection the cells were treated with 5 μg/ml Act D and either m.i. or infected with VSV at a multiplicity of 50 p.f.u./cell. VSV secondary transcripts from infected cells treated in the presence or absence of various concentrations of CCX were labelled with [35P]orthophosphate (25 μCi/ml) between 4 and 8 h p.i., and RNA was extracted as described (Johnson & Everett, 1986). RNA similarly extracted from m.i. cells had been labelled between 1 and 24 h.

**Preparation of RNA samples for agarose gel electrophoresis.** The yield of RNA was estimated using the absorbance at 260 nm. For electrophoresis, 38.75 μl of sample buffer [5 μl, 10 x MOPS running buffer; 8.75 μl formaldehyde (37%); 25 μl formamidomethanol] was added to 11.25 μl of RNA preparation and then 10 μl of formaldehyde loading buffer (1 mM-EDTA pH 8.0, 0.25% bromophenol blue, 0.25% syrene cyanol, 0.5% glycerol) was added. Electrophoresis was through 1:2 agarose/formaldehyde gels containing 1% MOPS running buffer and 0.5% formamide. Electrophoresis was at 5 V/cm until the dye had migrated halfway down the gel. After soaking in water overnight the gel was stained with ethidium bromide (250 μg/ml) for 45 min and then photographed under u.v. illumination (260 nm), allowing visualization of the total RNA loaded into each gel track. Finally, the gel was dried and placed against X-Omat S film to provide a autoradiograph.

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**MOPS (10 x) running buffer was prepared by adding 418 g MOPS to 800 ml diethylpyrocarbonate (DEPC)-treated H2O pH 7. DEPC-treated 3 m-sodium acetate (166 ml) and 20 ml DEPC-treated 0.5 M-EDTA pH 8 were then added and the total volume brought to 11 with DEPC-treated H2O; the buffer was filtered before use.

**Results**

The effect of CCX on the VSV infectivity in the CR and CA virus yields was measured by dose-response experiments which showed progressive reduction in infectious virus yield: 24 h treatment with 300 μM-CCX reduced the infectivity in the CA and CR components by 100- and 1000-fold respectively (Fig. 1). The greater sensitivity of the CR yield suggests that increasing the concentration of CCX either delays virus replication or slows the release of progeny VSV from the infected cell, or results in inactivation of CR virus at a faster rate than that of virus which remains associated with the cell. One-step VSV growth curves performed in the presence or absence of 300 μM-CCX (Fig. 2) showed that drug treatment abolishes production of infectious virus progeny. Therefore, both a delay in virus replication and slower release of progeny from infected cells can be discounted as explanations.

To investigate whether CCX inhibited VSV replication at a specific stage during the virus replication cycle, CCX, to 300 μM, was added to the growth medium of VSV-infected cells at 0, 2, 4, 6, 8, 10, 12, 14, 16 and 24 h p.i. All cultures were harvested at 24 h p.i. and the...
The effect of various concentrations of CCX on the 24 h yield of VSV particles, p.f.u. and particle:p.f.u. ratios for three independent experiments are detailed in Table 1. The data consistently showed that increasing the CCX concentration results in a progressive reduction in infectivity (p.f.u.), accompanied by a smaller decrease in the number of particles visible by electron microscopy (EM) and a consequent increase in the particle:p.f.u. ratio. Table 2 shows a further experiment in which the total yield was separated into its CR and CA components. With increasing CCX concentration the progeny virus infectivity decreased more rapidly in the CR component. This was not simply due to failure to release budding virus particles, but rather because of loss of virus quality, shown by the greatly increased particle:p.f.u. ratio. Particle:p.f.u. ratios could not be calculated for the CA component at 100, 200 and 300 μM-CCX as the number of virus particles visible by EM was too low to be measured.

To investigate whether CCX has a virucidal effect, suspensions of VSV prepared in PBS C₅ were incubated at 4 °C or 37 °C for 24 h with or without 300 μM-CCX and the infectivity was then assayed. Without the drug, VSV proved more stable at 4 °C than at 37 °C (79% and 17% of initial infectivity surviving respectively); the presence of 300 μM-CCX further reduced infectious VSV 10-fold and sevenfold respectively. In repeated experiments the magnitude of this virucidal effect varied somewhat, but never by greater than 10-fold. We conclude that the virucidal effect cannot by itself account for the greater sensitivity of CR VSV to CCX (Fig. 1 and Table 2).
To examine whether growth of VSV in the presence of CCX gave rise to progeny virions having alterations in structural proteins, the 24 h yield of [35S]methionine-labelled virus purified from control, or 150 μM- or 300 μM-CCX-treated cells was studied by SDS-PAGE. No significant differences were detected in the VSV L, G, N, NS or M proteins between control and CCX-treated virus and nor did these experiments provide evidence that CCX increased the amount of defective interfering VSV (data not shown).

We investigated the effect of CCX treatment on the abundance of cytoplasmic RNA transcripts in m.i. and VSV-infected cells. VSV primary transcripts are synthesized by a viral transcriptase which is a component of the virus particle (Marcus et al., 1971), whereas secondary transcripts, synthesized on progeny genomes by nascent transcriptase, depend upon protein synthesis. Primary and secondary transcripts are identical and consist of a leader RNA and five monocistronic mRNAs encoding five proteins, N, NS, M, G and L (Moyer et al., 1975; Moyer & Banerjee, 1975; Freeman et al., 1977; Rhodes et al., 1977). Transcriptional control regulates the quantity of each VSV protein made because VSV mRNAs are efficiently translated on host cell ribosomes (Villarreal et al., 1976). Thus, the effect of CCX on each VSV transcript can be correlated with the amount of each protein made. VSV secondary transcripts, cellular transcripts and polypeptides in m.i. and VSV-infected cells were labelled in parallel with [32P]orthophosphate or [35S]methionine between 4 and 24 h p.i. Act D was used to selectively suppress host cell RNA synthesis when labelling VSV secondary transcripts.

The effect of CCX on the synthesis of VSV secondary transcripts is shown in Fig. 4: with increasing CCX concentration VSV transcript synthesis progressively declined. Synthesis of the L transcript was no longer detectable with 100 μM-CCX (lane 2) and synthesis of the VSV transcripts was virtually undetectable with 300 μM-CCX (lane 4). Ethidium bromide staining of the gel showed that RNA loading differences could not account for the great reduction in the amount of VSV transcripts observed (data not shown). We concluded that treatment of infected cells with increasing CCX concentrations results in progressive dose-dependent reduction in the

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**Table 1. The effect of increasing concentration of CCX on the yield of VSV particles, infectious virus and particle : p.f.u. ratio after 24 h**

<table>
<thead>
<tr>
<th>CCX (μM)</th>
<th>Particles (x 10^9)</th>
<th>P.f.u. (x 10^6)</th>
<th>P:p*</th>
<th>Particles (x 10^9)</th>
<th>P.f.u. (x 10^6)</th>
<th>P:p</th>
<th>Particles (x 10^9)</th>
<th>P.f.u. (x 10^6)</th>
<th>P:p</th>
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<td>260</td>
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<td>519</td>
<td>10</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>10</td>
<td>210</td>
<td>47</td>
</tr>
<tr>
<td>100</td>
<td>0.75</td>
<td>2.2</td>
<td>340</td>
<td>0.5</td>
<td>3.9</td>
<td>128</td>
<td>0.7</td>
<td>2.16</td>
<td>324</td>
</tr>
<tr>
<td>200</td>
<td>&lt;0.1</td>
<td>0.05</td>
<td>ND</td>
<td>&lt;0.1</td>
<td>0.07</td>
<td>ND</td>
<td>&lt;0.1</td>
<td>0.07</td>
<td>ND</td>
</tr>
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</table>

* P:p, Particle : p.f.u. ratio.
† ND, Not determined.

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**Table 2. The effect of increasing concentrations of CCX on the yield of VSV particles, infectious virus and particle : p.f.u. ratio in the CA and CR components of the total virus yield after 24 h**

<table>
<thead>
<tr>
<th>CCX (μM)</th>
<th>Particles (x 10^9)</th>
<th>P.f.u. (x 10^6)</th>
<th>P:p*</th>
<th>Particles (x 10^9)</th>
<th>P.f.u. (x 10^6)</th>
<th>P:p</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0.14</td>
<td>10</td>
<td>14</td>
<td>10</td>
<td>280</td>
<td>357</td>
</tr>
<tr>
<td>50</td>
<td>0.7</td>
<td>40</td>
<td>17.5</td>
<td>10</td>
<td>190</td>
<td>52.6</td>
</tr>
<tr>
<td>100</td>
<td>&lt;0.1</td>
<td>6</td>
<td>ND†</td>
<td>0.8</td>
<td>2.2</td>
<td>363.6</td>
</tr>
<tr>
<td>200</td>
<td>&lt;0.1</td>
<td>1.0</td>
<td>ND</td>
<td>0.14</td>
<td>0.06</td>
<td>2333.3</td>
</tr>
<tr>
<td>300</td>
<td>&lt;0.1</td>
<td>0.1</td>
<td>ND</td>
<td>0.14</td>
<td>0.0016</td>
<td>87500</td>
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</tbody>
</table>

* P:p, Particle : p.f.u. ratio.
† ND, Not determined.
Effect of CCX on VSV replication

Fig. 4. The effect of CCX on cytoplasmic RNA levels in VSV-infected BSC-1 cells. The VSV L, G, N, M and NS cytoplasmic RNAs synthesized in infected cells either in the absence (lane 1), or the presence of 100, 200 or 300 μM-CCX (lanes 2, 3 and 4 respectively). Prior to infection and throughout cells were treated with 5 μg/ml Act D. RNA was labelled with [32P]orthophosphate between 4 and 24 h p.i. Equal quantities of RNA (determined by measuring absorbance at 260 nm) were applied to each lane.

synthesis of all VSV secondary transcripts. An analogous reduction in the levels of cellular 28S and 18S ribosomal transcripts synthesized was found for m.i. cell cultures treated with 300 μM-CCX (data not shown), indicating that ribosomal RNA synthesis is affected similarly.

Protein synthesis as total TCA-precipitable counts was measured and the effects of CCX, or CCX in combination with Act D, are given in Fig. 5. In the absence of Act D, increasing concentrations of CCX progressively reduced [35S]methionine incorporation into m.i. and VSV-infected cells; in the presence of Act D there was a CCX concentration related decrease of incorporation of label into infected cells, but not uninfected cells. As the main effect of Act D is to block cellular RNA synthesis, this again indicates that CCX affects VSV transcription. In parallel with the experiment in Fig. 4, we examined [35S]methionine-labelled extracts from VSV-infected cells treated with or without CCX and/or Act D by SDS-PAGE (Fig. 6).

CCX treatment induced the synthesis of cellular stress proteins (e.g. bands of 94K, 78K and 90K) in both m.i. and VSV-infected cells in the absence of Act D. In addition to the five recognized VSV gene products, L, G, N, NS and M, four additional virus-induced bands were seen (1, 2, 3 and 4); these are also apparent in gel profiles published by others (Evans et al., 1979; Paterson, 1983). The derivation of these polypeptides has not been studied and is unclear. The mobility of the G protein increased progressively with increasing CCX concentration (Fig. 6). Densitometry measurements from the gel showed that CCX alone resulted in L protein becoming undetectable with 200 μM-CCX, whereas N polypeptide progressively decreased and G protein synthesis appeared to be affected only by 300 μM-CCX. In contrast, NS and M protein synthesis were increased significantly by CCX. The presence of Act D reduced and then abolished the magnitude of the NS and M protein increases, whereas L, G and N proteins progressively decreased with CCX concentration.

In similar experiments investigating the effect of CCX on VSV primary transcription, no viral transcripts were detected. As all viral proteins were made in substantial amounts (data not shown) it seems unlikely that CCX has a significant effect on VSV primary transcription.

In VSV-infected cells treated with 300 μM-CCX (Fig. 5) [35S]methionine incorporation into protein was only reduced to 61% of the control level whereas the number of VSV particles decreased 100-fold (Table 1 and 2). Thus, the data argue for CCX also having a strong inhibitory effect on VSV particle assembly.

Glycosylation of cellular (BHK and Flow 2002) and HSV glycoproteins is known to be strongly inhibited by
CCX and CBX (Dargan & Subak-Sharpe, 1986a, b), and Mon also inhibits glycosylation in the Golgi apparatus (Ledger & Tanzer, 1984; Boss et al., 1984). Tun blocks addition of the first N-linked oligosaccharides to the nascent glycoprotein in the endoplasmic reticulum (Tkacz & Lampen, 1975). Therefore, we compared the effects of CCX, Mon and Tun on glycosylation in m.i. and VSV-infected cells. M.i. and VSV-infected cells were treated in parallel with CCX, Mon or Tun and labelled (8 to 24 h) with $[^{35}\text{S}]$methionine or $[^{14}\text{C}]$glucosamine. The data obtained with m.i. cells are not given, but briefly they showed that Tun induces the synthesis of additional prominent stress-related proteins of 120K and 35K, whereas Mon fails to induce any stress proteins; without drug, four major and many minor polypeptide bands label with $[^{14}\text{C}]$glucosamine; CCX produces a progressive reduction in the intensity of labelling of all these bands and a drug dose-dependent increase in the electrophoretic mobility of the major bands; Mon treatment does not significantly affect the intensity of labelling of any band, although the electrophoretic mobility of two bands is increased; Tun treatment virtually abolishes $[^{14}\text{C}]$glucosamine labelling.

After labelling VSV-infected cells with $[^{35}\text{S}]$methionine it was shown that CCX caused a progressive reduction in proteins L and NS, but an increase in G and M proteins with no apparent effect on the N protein (Fig. 7a). Densitometry demonstrated that 300 μM-CCX caused an increase in G and M proteins by 385% and 575% respectively (Fig. 7b). (Note that G and NS proteins behave differently to Fig. 6 in this experiment.) In cultures treated with Mon, the amounts of L, N and NS proteins were virtually unchanged whereas those of G and M proteins were substantially increased (Fig. 7a and b). With Tun, the amounts of L, N and NS proteins appeared to be unchanged, whereas the G and M
Effect of CCX on VSV replication

Fig. 7. The effect of 24 h treatment with CCX, Mon or Tun on the synthesis and glycosylation of VSV polypeptides. (a) The infected (m.o.i. 5 p.f.u./cell) cell polypeptides labelling with [35S]methionine between 8 and 24 h p.i. in cultures treated either in the absence (lane 1) or presence of CCX (25, 50, 75, 100, 150, 200 or 300 μM; lanes 2, 3, 4, 5, 6, 7 and 8 respectively), Mon (5 or 10 μM; lanes 9 and 10 respectively) or Tun (2 μg/ml; lane 11). The L, G, N, NS and M polypeptides, virus-induced proteins 1, 2, 3 and 4, and the different electrophoretic mobilities of the G glycoprotein (G, mature form; Gp, partially processed; Go, unglycosylated precursor) are indicated. (b and c) The effect of CCX (c), and Mon and Tun (b) on the amount of the VSV-specified proteins synthesized in drug-treated cells. Densitometric quantification of the L (●), N (○), NS (△), G (□) and M (▲) polypeptides (see a, lanes 1 to 11). The amount of each protein is presented as a percentage of the amount present in the drug-free control, the areas under the peaks of which were L, 1180; N, 3546; NS, 1137; G, 757; and M, 548. (d) The infected (m.o.i. 5 p.f.u./cell) cell polypeptides labelling with [14C]glucosamine between 8 and 24 h p.i. in cultures treated either in the absence (lane 2) or presence of CCX (25, 50, 75, 100, 150, 200 or 300 μM, lanes 3, 4, 5, 6, 7, 8 and 9, respectively), Mon (5 or 10 μM, lanes 10 and 11, respectively) or Tun (2 μg/ml, lane 12). Lane 1 shows the m.i. polypeptides labelling with [14C]glucosamine.

proteins increased in amount (Fig. 7a and b). The different responses of the five virion proteins demonstrate that the effect is on virion component pools and not mature particles. The electrophoretic mobility of the G protein band was increased with each drug, suggesting an effect on oligosaccharide processing. In extracts treated with CCX (200 μM and 300 μM) and Mon (5 μM and 10 μM) the G protein band ran to equivalent positions in the gel, but with Tun it exhibited even greater mobility, pinpointing the unglycosylated precursor position for the G glycoprotein (Fig. 7a). The VSV G protein is the major band that labels with [14C]glucosamine in infected cell extracts (Fig. 7d) and the intensity of this band increased markedly with increasing CCX concentration (to 200 μM). This supports the [35S]methionine data (Fig. 7a). In contrast to the strong inhibition of glycosylation in HSV-infected BHK and Flow 2002 cells (Dargan & Subak-Sharpe, 1986a, b), the processing
of the VSV G protein in BSC-1 cells was not inhibited by concentrations of CCX as high as 200 μM, although it began to be affected by 300 μM (compare Fig. 7a, lane 8 with d, lane 9). With 5 μM- and 10 μM-Mon the mobility of the G protein band labelled with [35S]methionine was higher, but the intensity of [14C]glucosamine labelling was higher (Fig. 7a, lanes 9 and 10 and d, lanes 10 and 11). Tun blocked all addition of [14C]glucosamine to the glycoprotein (Fig. 7d, lane 12). Bearing in mind the results shown in Fig. 4 and 7, we concluded that the intracellular stability of the G protein is enhanced by CCX, Mon or Tun treatment. The electrophoretic mobility of the G protein increased suggesting reduced oligosaccharide processing with both CCX and Mon, whereas Tun blocks processing and only the unglycosylated G protein precursor is produced.

Treatment with CCX, Mon and Tun appeared to result in similar relative responses at the polypeptide level (Fig. 7), so we compared the effects of the three drugs on VSV particle number, infectious virus yield and the particle: p.f.u. ratio. Treatment of infected cells with 150 μM- or 200 μM-CCX increased the VSV particle: p.f.u. ratio at least 10-fold (Table 3), whereas Mon and Tun had no significant effect. This indicates (i) that glycosylation of the VSV G protein is not required for the production of fully infectious virus, consistent with the findings of Gibson et al. (1978); (ii) that assembly in the presence of CCX (but not Mon or Tun) results in reduced quality of the virus progeny; and (iii) reduced particle yield correlates with reduced glycosylation: this may be due to the drug’s direct effect on G protein glycosylation or a drug effect on another function essential for particle formation.

Table 3. The effect of CCX, Mon and Tun on the yield of VSV particles, infectious virus and particle: p.f.u. ratio from drug-treated cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Particles (× 10^2)</th>
<th>P.f.u. (× 10^2)</th>
<th>P:p*</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>13</td>
<td>450</td>
<td>29</td>
</tr>
<tr>
<td>CCX (μM)</td>
<td>150</td>
<td>0.85</td>
<td>2.5</td>
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<tr>
<td></td>
<td>200</td>
<td>0.143</td>
<td>0.48</td>
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<tr>
<td></td>
<td>300</td>
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<td>0.023</td>
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<tr>
<td>Mon (μM)</td>
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<td>0.143</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.143</td>
<td>3.6</td>
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</table>

* P:p, Particle: p.f.u. ratio.
†ND, Not determined.

Discussion

CCX is a potent inhibitor of VSV replication in BSC-1 cells. The 1000- to 10000-fold reduction in infectious VSV yield (Fig. 1) in cultures treated for 24 h with 300 μM-CCX resembles the reduction in HSV infectious yield with this concentration of drug (Dargan & Subak-Sharpe, 1985). The data concerning VSV particle number and particle: p.f.u. ratio demonstrate additional similarities in the antiviral effect of CCX for these two CCX-1 class viruses (Table 1; Dargan & Subak-Sharpe, 1985); although one is an RNA and the other a DNA virus, the antiviral effect of CCX in each case operates both through inhibition of particle assembly and by a reduction of quality (average infectivity) of the virus progeny. SDS–PAGE studies have correlated CCX-induced loss of HSV infectivity with multiple quantitative changes in the HSV set of structural proteins (Dargan & Subak-Sharpe, 1986a). The reduced quality of the infectious HSV virions made in the presence of CCX is supported by the demonstration of their greater thermostability (Dargan & Subak-Sharpe, 1986a). With VSV we could not detect any differences in the polypeptide profile of virions produced with or without CCX. The virucidal effect of CCX reduces both VSV and HSV infectivity approximately 10-fold in 24 h at 4°C or 37°C (Dargan & Subak-Sharpe, 1985). The mechanism by which CCX elicits its virucidal effect has not been investigated; however, the drug is highly lipophilic (Symons & Parke, 1980) and probably interacts with the VSV and HSV envelope, so affecting adsorption and/or penetration.

The antiviral activity of CCX against VSV and HSV operates throughout the virus replication cycle, indicating that the drug does not accomplish its effect through the specific inhibition of any single essential virus gene product (Fig. 3; Dargan & Subak-Sharpe, 1985). VSV primary transcription does not appear to be significantly affected, but we found a rapid CCX dose-dependent reduction in the levels of all VSV secondary transcripts (Fig. 4). Possible explanations are that the drug might block transport of RNA, inhibit the transcription process itself, or result in the synthesis of mRNAs with altered stability. As VSV replication is completely cytoplasmic, impaired transport of RNA cannot explain the levels of VSV transcripts, although it could account for the levels of cellular transcripts. CCX could influence the activity of the transcription process indirectly through mobilizing the cellular stress response (Fig. 6), which involves vigorous stress protein mRNA transcription, and a concomitant inhibition of transcription and translation of other mRNAs (Ashburner & Bonner, 1979). However, in cultures treated with both CCX and Act D, which abolishes the stress response, VSV
secondary transcripts are reduced, so stress cannot furnish the full explanation. Nevertheless, under the usual conditions of infection, the CCX-induced stress response could play some role in reducing the levels of cell- and virus-specified transcripts. CCX did not affect the phosphorylation of cellular or VSV polypeptides detectably (data not shown). One possible explanation for the effect of CCX on the levels of cellular and VSV transcripts stems from the work of Mukherjee & Simpson (1987) on indomethacin. CCX and indomethacin could have equivalent effects on a cell membrane-associated transcription factor required for the transcriptase activity of the VSV nucelocapsid, as both CBX (Roberts & Taylor, 1980) and indomethacin alter the biosynthesis of prostaglandins which modify the transcription factor. Other possible explanations for the effect of CCX on cytoplasmic transcripts include activation of RNases, activation of proteases resulting in degradation of the transcriptase complex, or binding of the drug to the transcriptase complex itself, thereby modifying or inhibiting its function. Further work is necessary to resolve these questions.

The amounts of individual VSV polypeptides found in CCX-treated cells varied; L and N (and NS) proteins were decreased whereas M (and G) protein increased in amount despite a reduction in the levels of their transcripts. (There was some interexperimental variability as far as G and NS proteins are concerned. This may have been due to the physiological differences between the BSC-1 cell cultures used.) The precise mechanism of action of CCX, which results in differential accumulation of some VSV proteins, notably M protein, is of interest and needs further investigation. Possible explanations include differential stability of mRNAs and/or preferential translation of some mRNAs; however, a more likely explanation is that the levels of VSV proteins reflect the stability of individual proteins or protein complexes under conditions which restrict virus assembly. The amounts of individual VSV polypeptides observed in CCX-treated cell extracts will reflect two superimposed effects – the first on transcription/translation and the second on protein pool stability.

Glycosylation of cellular and VSV glycoproteins was compared in cells treated with CCX, Mon or Tun. CCX and Mon produced a similar small increase in the electrophoretic mobility of VSV glycoprotein G, indicative of an effect of drug late in the maturation of the oligosaccharide chain, possibly in the trans stacks of the Golgi apparatus or in post-Golgi transport vesicles. Tun produced a much larger increase in the electrophoretic mobility of G protein and a complete inhibition of [14C]glucosamine labelling of G protein (Fig. 7a, d).

Johnson & Schlesinger (1980) have shown that Mon causes dilation of Golgi apparatus membranes and that transport of the VSV G protein from the Golgi apparatus to the plasma membrane, at which VSV assembly normally occurs (Simons & Garoff, 1980), is impaired in infected BHK and chick embryo fibroblast cells. Thus, retention of the G protein at the Golgi apparatus suppresses VSV virion assembly. All three drugs, CCX, Mon and Tun, induce the accumulation of the G and M proteins within infected cells. The VSV matrix protein, M, is thought to play a pivotal role in VSV assembly, mediating interactions between the nucleocapsid and G protein (Simons & Garoff, 1980). We propose that the accumulation of G and M proteins within infected cells treated with CCX is a consequence of a drug-induced restriction of virus assembly.

Since VSV grown in the presence of Mon and Tun retains full infectivity, it is clear that glycosylation of the G protein is not important for infectivity (Gibson et al., 1978). It follows that loss of virus quality when grown in the presence of CCX is not due to the effect of the drug on glycosylation, but some other function involved in VSV particle assembly. In the case of treatment with CCX or Mon, the inhibited function of the Golgi apparatus which affects particle formation, is not processing of the glycoprotein, but transport of the G protein to and its insertion into the plasma membrane. This restricts the total number of VSV particles assembled. The transport of G protein in cultures treated with Tun must be affected similarly.

We conclude that VSV replication is extremely sensitive to treatment of host cells with CCX. The result is an at least 100-fold reduction in the number of particles assembled, further exacerbated by loss of quality of progeny virus particles. The known effect of CCX on glycoprotein processing is shown not to be involved in the loss of virus quality, implicating CCX-mediated impairment of another function required for VSV assembly as the cause. The effect of CCX on the transport function of the Golgi apparatus, and the effect of the drug on VSV secondary transcription and possibly on the stability of the protein products is considered to be responsible for the great reduction in the number of particles assembled. Lastly, CCX has a further virucidal effect on suspended mature VSV particles which results in 10% survival after a 24 h period.

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References


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