Differentiation of herpes simplex virus-induced fusion from without and fusion from within by cyclosporin A and compound 48/80

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T Treating strains of herpes simplex virus (HSV) in culture with either cyclosporin A or compound 48/80, allowed the strains to be divided into two groups. Group 1 contains the strains ANG and HFEM of HSV-1 and Lux syn (HSV-2) producing fusion from within (FFWI) and fusion from without (FFWO). Cyclosporin A fails to inhibit both types of fusion at concentrations up to 100 μM. Strains ANG and HFEM belong to the syn 3 marker locus group identified for HSV-1. Group 2 contains all other fusion-producing strains of HSV tested so far. Cyclosporin A inhibits FFWI at concentrations as low as 10 to 20 μM. These strains belong to the syn locus marker groups 1, 2, 4 and 5. From the fact that mutations in glycoprotein B belong to the syn 3 marker group we conclude that glycoprotein B is of major importance for FFWO. Compound 48/80 also differentiates between these two groups of viruses. O-Acetyl cyclosporin A is unable to inhibit FFWI induced by group 2 viruses; in contrast, cyclosporin H and the Ca²⁺ ionophore A23187 exert inhibition effects similar to those exerted by cyclosporin A. We conclude from the effects of these compounds that binding properties of the OH group of cyclosporin A and an increase of Ca²⁺ ions may be preconditions for the observed effects. Binding of cyclosporin A to cyclophilin does not appear to be responsible for these effects.

Introduction

Cytopathic effects induced by herpes simplex virus (HSV) are manifested by either rounding or fusion of cells. This fusion has been named fusion from within (FFWI) because fusion is induced during the course of virus replication. FFWI can be blocked by inhibitors of transcription and translation and can be detected 3 to 4 h after infection (Falke et al., 1985). Up to six loci of HSV may be involved in fusion processes (Tognon et al., 1988; Spear, 1987), and mutations in one of these loci might induce fusion capacity. Mutations in the syn 3 locus have been attributed to the gene for glycoprotein B (gB), which is involved in penetration and fusion (Weizhong et al., 1988).

Some time ago, we reported on fusion from without (FFWO) induced by certain strains of HSV-1 (Falke et al., 1985). FFWO is produced by the virus particles themselves 30 to 45 min after infection and cannot be inhibited by inhibitors of transcription or translation (Falke et al., 1985). Furthermore, it was possible to show that strains of HSV-1 and -2 producing FFWI could be divided into two groups: strains producing FFWI and FFWO, and strains exhibiting FFWI activity only (Walev et al., 1991). Recently, McKenzie et al. (1987) observed inhibition of FFWI produced by strain MP (HSV-1) after addition of cyclosporin A (CyA) without any influence on penetration of this HSV strain. CyA is thought to bind strongly to membranes and presents its polar hydroxyl group outwards from the lipophilic bilayer into the hydrophilic phases (Epand et al., 1987; LeGrue et al., 1983). It also binds to cyclophilin (Handschumacher et al., 1984) and enhances the Ca²⁺ ion influx (Nicchitta et al., 1985). Compound 48/80 (Cpd 48/80) selectively inhibits FFWI induced by HSV-1 strain IES, without altering the synthesis of infective particles (Falke & Kahl, 1970). It also moderately reduces the penetration of HSV particles (Falke & Netter, 1969). Cpd 48/80, moreover, was shown to inhibit non-competitively the cholin kinase (Just & Falke, 1974), but in the dose range used did not affect DNA, RNA and protein synthesis (Falke & Kahl, 1971); it also binds to anions and activates GTP-binding proteins (Mousli et al., 1990). We now report on experiments demonstrating that CyA and Cpd 48/80 allow differentiation of the fusion events of FFWI-positive strains of HSV-1 and -2 into two groups. One group of HSV strains producing only FFWI is strongly inhibited by CyA and
Cpd 48/80, whereas three strains of the other group, which are positive for FFWO, are not inhibited by CyA and only moderately by Cpd 48/80.

Methods

Viruses. We used the HSV-1 strains ANG (Falke et al., 1985), HFEM (Walev et al., 1991), IES (Falke & Kahl, 1971), MP (Heeg et al., 1986), Len (Heeg et al., 1986), 17 TK 1301 (Sanders et al., 1982), L4-1 and L3-28 (Falke et al., 1985). Strains KOS-804 (syn) and KOS-78R (Little & Schaffer, 1981) were provided by P. Schaffer, Boston, Mass., U.S.A., Sharpe (Glasgow, U.K.) and HSV-2 strains Lux syn and US-A12 by K. E. Schneweis (Bonn, Germany). Viruses were grown in Vero cells maintained in roller bottles at 37 °C. Titrations were done as described earlier (Falke et al., 1985).

Cell culture and medium. Vero cells (2 x 10⁴ to 4 x 10⁴) were seeded into 96-well Nunclon plates and kept overnight before the FFWO tests. Medium 199 was used with 5% selected, inhibitor-free foetal calf serum (Walev et al., 1991).

FFWO and FFWI assay. In order to produce FFWO, subconfluent Vero cell monolayers in 96-well plates were infected with 100 μl of virus at a concentration of 5 x 10⁷ to 10 x 10⁷ p.f.u. per ml. FFWO was assessed at 45 to 60 min after incubation at 37 °C as 4+ (100%), 3+ (75%), 2+ (50%), 1+ (25%) or no fused cells. FFWI was induced with 5 x 10² to 1 x 10⁵ p.f.u. per well to give a 4+ level of fusion at 18 h of incubation at 37 °C.

Substances. CyA was kindly provided by Sandoz AG. CyA is a cyclic undecapeptide; amino acid 1 contains a hydroxyl group and amino acid 11 is a methyl-valine (Wenger, 1986). It was dissolved in DMSO or ethanol at a concentration of 20 mM and diluted as necessary. For FFWO tests, CyA was added 12 h before, and for FFWI tests 2 h after infection as done previously by McKenzie et al. (1987). As controls, suitable DMSO or ethanol dilutions were run. CyH has in position 11 a D-methyl-valine; esterification of an acetyl group to the OH group on amino acid 1 gives rise to O-acetyl CyA (Wenger, 1986). Both drugs were kindly provided by Sandoz AG. CyH and the Ca²⁺ ionophore A23187 (Sigma) were dissolved as for CyA; O-acetyl CyA was suspended by ultrasonication in ethanol as formulated by Sandoz AG. The calmodulin inhibitor R24571 supplied by Sigma was dissolved in ethanol. Cpd 48/80 is a mixed oligomer of p-methoxy-N-methyl phenylethylamine cross-linked by formaldehyde (Falke & Kahl, 1971). All compounds were added uniformly 2 h after infection (FFWI assay) to avoid any influence on penetration. No toxicity for cells was observed after addition of any of these substances at the indicated concentrations at a pH of 7.0 for 24 h.

Results

Effect of CyA on HSV-induced FFWI

Our experiments were designed to differentiate between HSV strains producing FFWI. Tests were read 18 h after infection, a time at which there was a 4+ fusion reaction in the controls. It can be seen (Fig. 1a) that the FFWI reaction produced by strains ANG, HFEM and Lux syn was not inhibited by 100 μM-CyA. In contrast, the FFWI activity induced by strains 17 TK 1301, MP, KOS-804 syn and IES was completely blocked at 20 to 40 μM-CyA; instead cell rounding was observed. Identical results have been obtained with strains Len, L4-1, L3-2S, 17 syn and US-A12 (data not shown). Thus, it was possible to differentiate between strains of HSV active in FFWI into two groups; one group produced FFWI (ANG, HFEM and Lux syn) in the presence of CyA, and
another group produced no FFWI. It should be added that CyA, CyH and the Ca\(^{2+}\) ionophore A23187 at the effective concentrations reduce the production of p.f.u. after 18 to 24 h of incubation by 50 to 70\% (data not shown).

**Influence of CyA on HSV-induced FFWO**

Having shown that CyA does not inhibit FFWI induced by strains ANG, HFEM and Lux syn, we tested the influence of this drug on FFWO produced by these strains. CyA was added to the cell cultures 12 h before high multiplicity infection for the FFWO assay. From Fig. 1(b) it can be seen that CyA at the indicated concentrations does not inhibit FFWO induced by these strains.

**Effect of Cpd 48/80 on HSV-induced FFWI**

Using Cpd 48/80 (instead of CyA), which inhibits FFWI induced by strains IES (Falke & Kahl, 1971), we repeated the FFWI and FFWO experiments. It can be seen from Fig. 1(c) that FFWI induced by the FFWO-producing strains ANG, HFEM and Lux syn is inhibited only at high concentrations of Cpd 48/80. In comparison, those strains producing only FFWI were inhibited, with regard to fusion, at much lower concentrations of Cpd 48/80. Therefore Cpd 48/80, as well as CyA, allows

### Table 1. Effect of CyA and analogues, ionophore A23187 and calmodulin inhibitor R24571 on HSV-induced FFWO and FFWI

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration ((\mu)M)</th>
<th>Toxicity</th>
<th>ANG FFWO*</th>
<th>ANG FFWI†</th>
<th>IES FFWI</th>
<th>KOS-804 FFWI</th>
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<tbody>
<tr>
<td>CyA</td>
<td>40–100</td>
<td>–</td>
<td>4+†</td>
<td>4+</td>
<td>CR†</td>
<td>CR</td>
</tr>
<tr>
<td>CyH</td>
<td>40–100</td>
<td>–</td>
<td>4+</td>
<td>4+</td>
<td>CR</td>
<td>CR</td>
</tr>
<tr>
<td>O-Acetyl CyA</td>
<td>40–100</td>
<td>–</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>A23187</td>
<td>2–10</td>
<td>–</td>
<td>4+</td>
<td>4+</td>
<td>CR</td>
<td>CR</td>
</tr>
<tr>
<td>Cpd R24571</td>
<td>2–10</td>
<td>–</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
</tr>
</tbody>
</table>

* FFWO, high m.o.i., duration of experiment was 60 min.
† FFWI, low m.o.i., duration of experiment was 24 h.
‡ 4+, One hundred per cent fusion of cells after 60 min or 24 h.
§ CR, Cell rounding.
‖ Toxicity above 10 \(\mu\)M after 24 h of incubation.

### Table 2. Influence of CyA and Cpd 48/80 on FFWI and FFWO induced by HSV

<table>
<thead>
<tr>
<th>HSV type and strain</th>
<th>Syn locus</th>
<th>Map coordinates of syn mutation</th>
<th>FFWI CyA (40 (\mu)M)</th>
<th>FFWO CyA (100 (\mu)M)</th>
<th>FFWI Cpd 48/80 (50 (\mu)g)</th>
</tr>
</thead>
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<tr>
<td>1 MP</td>
<td>Syn 1</td>
<td>0.68-0.82 (Ruyechan et al., 1979)</td>
<td>CR*</td>
<td>NO†</td>
<td>CR</td>
</tr>
<tr>
<td>MP</td>
<td>Syn 2</td>
<td>0.724-0.747 (Little &amp; Schaffer, 1981)</td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>KOS-78R</td>
<td>Syn 3</td>
<td>0.3-0.42 (Ruyechan et al., 1979)</td>
<td>4+§</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>HFEM</td>
<td>Syn 3</td>
<td>0.345-0.350 (Weise et al., 1987)</td>
<td>4+</td>
<td>4+</td>
<td>3+</td>
</tr>
<tr>
<td>ANG</td>
<td>Syn 4</td>
<td>0.040-0.064 (Little &amp; Schaffer, 1981)</td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>KOS-804 syn</td>
<td>Syn 5</td>
<td>0.296-0.317 (Sanders et al., 1982, Tognon et al., 1988)</td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>17 TK 1301</td>
<td>Syn 5</td>
<td>0.296-0.317 (Sanders et al., 1982, Tognon et al., 1988)</td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
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<tr>
<td>L4-1</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>L3-2s</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
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<tr>
<td>IES</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
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<tr>
<td>Len</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>17 syn</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>2 Lux syn</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
<tr>
<td>US-A12</td>
<td>?</td>
<td></td>
<td>CR</td>
<td>NO</td>
<td>CR</td>
</tr>
</tbody>
</table>

* CR, Cell rounding.
† NO, Strain does not produce FFWO.
‡ 39 °C.
§ 2+, 3+, 4+: 50, 75 or 100\% fused cells.
differentiation between the above-mentioned groups of FFWI-positive strains of HSV.

Although Cpd 48/80 did inhibit FFWI moderately at higher concentrations (75 to 200 μg/ml), Cpd 48/80 also inhibits penetration (Falke & Netter, 1969). Therefore these results cannot be ascribed completely to a FFWI blocking effect of this drug (data not shown).

In Fig. 2 the different types of c.p.e. can be seen. Fig. 2(a) shows FFWI as induced by strain IES, whereas Fig. 2(b) illustrates cell rounding as observed following addition of 20 μM-CyA. Fig. 2(c) and (d) represent FFWI induced by strain ANG in the absence and presence of 100 μM-CyA, respectively.

Effect of CyA and analogues and of compounds A23187 and R24571 on HSV-induced cell fusion

Further experiments using analogues of CyA, the Ca^{2+}-ionophore A23187 and the calmodulin inhibitor R24571 were designed to obtain more information on the mechanism of the effect(s) of CyA (Table 1). Infection and addition of substances were done as described in Methods. CyH and A23187 exhibit effects like CyA. O-acetyl CyA and R24571 do not inhibit FFWI by strains IES and KOS-804.

Correlation of syn markers to CyA sensitivity

Up to now, six syn markers have been recognized (Tognon et al., 1988; Spear, 1987). We tried therefore to correlate CyA sensitivity of fusion to these syn markers. FFWO and FFWI as induced by strains ANG, HFEM (HSV-1) and Lux syn (HSV-2) could not be influenced by CyA.

The fusion activity of strains ANG and HFEM has been mapped to the syn 3 locus (Table 2). The strains MP, KOS-78R, KOS-804, 17 TK 1301 belong to the syn marker groups 1, 2, 4 and 5. Thus it emerges that the FFWO-positive strains ANG and HFEM of HSV-1 belong to the syn 3 marker group, a group of fusion-producing viruses which are not sensitive to CyA. The fusion activity of the remainder of the strains has not been mapped (Table 2).
Discussion

Fusion of cells depends on properties of the membrane (Roos et al., 1990), but it can only be induced by certain strains of HSV. Moreover we have to differentiate between FFWI and FFWO generated by HSV (Falke et al., 1985). Recently we have shown that penetration can be dissociated from FFWO by some substances: antibody and low pH treatment as well as addition of Zn²⁺ ions inhibit penetration, whereas treatment with N-ethylmaleimide (NEM) and cytochalasin B blocks the fusion process itself about 10 min later (Walev et al., 1991). NEM inhibits FFWO, possibly by binding to a cellular fusion protein; cytochalasin B causes disruption of the microfilaments involved in FFWO (Walev et al., 1991). Because we were looking for cellular constituents or functions participating in FFWO or FFWI, we used CyA and Cpd 48/80 as potential inhibitors of fusion processes. These experiments enabled us to identify FFWO-producing strains of HSV.

The fusion activities of one group of viruses (strains ANG, HFEM of HSV-1 and Lux syn of HSV-2), both FFWO and FFWI, were not inhibited by CyA. However CyA was able to inhibit fusion by the other group of strains of HSV-1 or -2 able to induce only FFWI. Thus we were able to recognize and differentiate between FFWO-positive strains of HSV by the addition of CyA. From Table 2 it can be seen that strains ANG and HFEM belong to the syn 3 group of HSV-1. Therefore it is assumed that gB plays a major role in FFWO generation. This has also been shown using recombinant viruses (D. Falke et al., unpublished results). We are now studying by sequence analysis the gB{s of strains of HSV distinguishable in regard to their fusion-inducing capacities.

Cpd 48/80 also allows a similar differentiation of these strains. The inhibition kinetics, however, are somewhat different. FFWI induced by the three FFWO-positive strains is inhibited only at high concentrations whereas strains inducing only FFWI are inhibited even at low concentrations. This indicates another mechanism of blockage when compared to CyA. So far as the action of Cpd 48/80 is known, this should be due either to binding of this drug to anionic moieties necessary for fusion events, or to the involvement of Cpd 48/80 in GTP-protein activation. We have no evidence to suggest the latter possibility (I. Walev, unpublished results). In the case of CyA, differential binding activity of the OH group of this substance to the protein(s) participating in the fusion process should be considered, particularly as O-acetyl CyA exhibits little binding activity. This indicates a role of the OH group in amino acid 1 for its effect. Regarding the FFWO-blocking effect of Ca²⁺ ionophore A23187, we propose a central role of Ca²⁺ ions during induction of FFWI. CyA also enhances the influx of Ca²⁺ ions (Nicchitta et al., 1985); therefore it may be possible that CyA exerts its effect on FFWI not only by reactions with its OH group but also by its influence on Ca²⁺ metabolism. This effect may be exhibited at an earlier step than Ca²⁺-binding by calmodulin (Hess & Colombani, 1986). The binding of CyA to cyclophilin does not appear to be necessary for its effects on FFWI, because CyH, which does not bind to this cellular protein (Handscharumacher et al., 1984), prevents FFWI.

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References


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