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Herpes simplex virus triggers activation of calcium-signaling pathways

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The cellular pathways required for herpes simplex virus (HSV) invasion have not been defined. To test the hypothesis that HSV entry triggers activation of Ca^{2+}-signaling pathways, the effects on intracellular calcium concentration ($[Ca^{2+}]_i$) after exposure of cells to HSV were examined. Exposure to virus results in a rapid and transient increase in $[Ca^{2+}]_i$. Pretreatment of cells with pharmacological agents that block release of inositol 1,4,5-triphosphate (IP_3)–sensitive endoplasmic reticulum stores abrogates the response. Moreover, treatment of cells with these pharmacological agents inhibits HSV infection and prevents focal adhesion kinase (FAK) phosphorylation, which occurs within 5 min after viral infection. Viruses deleted in glycoprotein L or glycoprotein D, which bind but do not penetrate, fail to induce a $[Ca^{2+}]_i$ response or trigger FAK phosphorylation. Together, these results support a model for HSV infection that requires activation of IP_3-responsive Ca^{2+}-signaling pathways and that is associated with FAK phosphorylation. Defining the pathway of viral invasion may lead to new targets for anti-viral therapy.

Introduction

Invasion of cells by herpes simplex virus type 1 or type 2 (HSV-1 and HSV-2) requires binding of the envelope glycoprotein C (gC) and/or glycoprotein B (gB) to heparan sulfate receptors, engagement by glycoprotein D (gD) of one of several coreceptors (herpes virus entry mediators), fusion of the viral envelope with the cell plasma membrane, and delivery of the viral capsid into the cell cytoplasm (Morgan et al., 1968; Herold et al., 1991, 1994; Spear et al., 2000; Spear and Longnecker, 2003). Most reports have focused on identifying the receptors and ligands required for viral binding and entry. However, the cellular pathways that participate in this process have not been defined. Knowledge of these pathways may facilitate development of novel strategies to prevent infection.

For most cell types, HSV entry (defined as fusion of the viral envelope with the cell plasma membrane and delivery of the viral capsid into the cell cytoplasm) is resistant to agents such as amantadine, chloroquine, and trifluoperazine, whose actions are known to alter endocytic pathways (Wittels and Spear, 1991; Nicola et al., 2003). Fusion requires the concerted action of gD, oligomers of gB, and heterodimers of glycoproteins H and L (gH–gL; Spear and Longnecker, 2003). Cells transiently or permanently expressing gD, gB, and gH–gL (Turner et al., 1998; Muggeridge, 2000) can induce fusion. Deletion of any one of these glycoproteins results in loss of penetration (Cai et al., 1987; Johnson and Ligas, 1988; Ligas and Johnson, 1988; Forrester et al., 1992; Hutchinson et al., 1992). However, the mechanism by which interactions between these viral glycoproteins and the cell surface trigger fusion is not defined. No specific fusion domains in HSV envelope glycoproteins have been identified.

We observed that HSV entry is associated with tyrosine phosphorylation of cellular proteins (Qie et al., 1999). Using immunoprecipitation and Western blotting, we found that several host cell proteins become tyrosine phosphorylated within 5–10 min after exposure to either HSV-1 or HSV-2. However, no phosphorylation was detected when cells were exposed to a virus deleted in gL that binds but fails to penetrate. Phosphorylation was restored when the gL deletion virus was grown on complementing gL-expressing cells. Viral infection and gene expression were inhibited by tyrphostin B46, a protein tyrosine kinase inhibitor that prevents the phosphorylation of cellular proteins (Qie et al., 1999).

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Key words: focal adhesion kinase; viral entry; membrane fusion; IP_3; tyrosine phosphorylation

Abbreviations used in this paper: 2-APB, 2-aminoethoxydiphenylborate; $[Ca^{2+}]_i$, intracellular calcium concentration; Δ, mean change; gC, gB, gD, gH, gL, glycoprotein C, B, D, H, or L, respectively; HCMV, human cytomegalovirus; HI, heat-inactivated; HSV, herpes simplex virus; IP_3, 1,4,5-triphosphate; moi, multiplicity of infection; odu, optical densitometry units; pfu, plaque-forming units; Tg, thapsigargin; VSV, vesicular stomatitis virus.

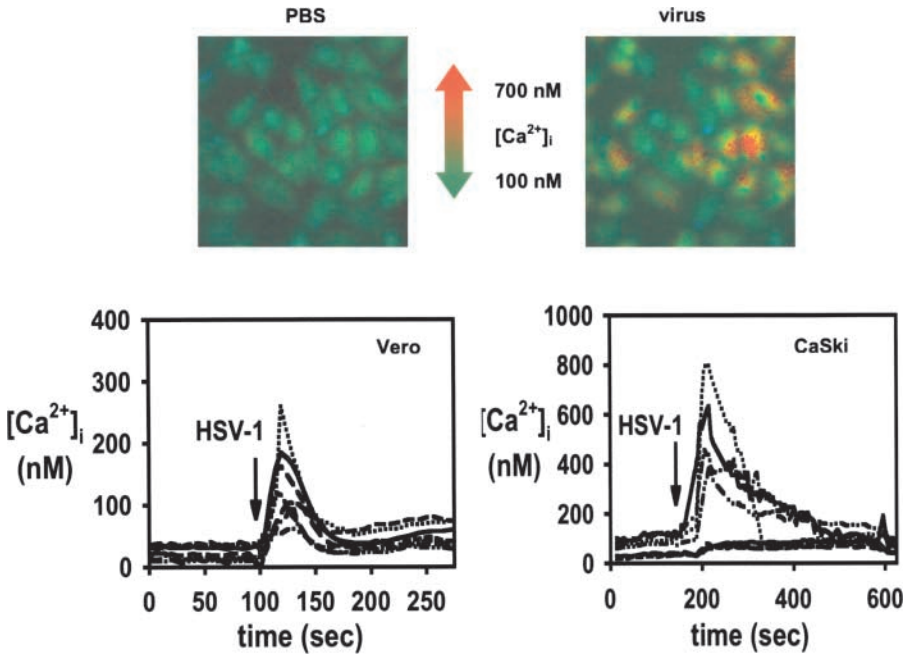

Figure 1. **The $[Ca^{2+}]_i$ response to HSV.** Vero or CaSki cells were loaded with the Ca^{2+} indicator dye Fura-2, exposed to HSV-1(KOS) (moi ~1.0), and changes in $[Ca^{2+}]_i$ were monitored. Representative fields from Vero cells as viewed under the microscope before (left) and ~20 s after exposure to virus (right) are shown in the top panels, and the results from a single experiment for each cell type in which 5–7 individual cells were monitored are shown graphically below.

These analyses suggest that phosphorylation pathways are activated in response to initiation of fusion and may be required for infection.

Several reports have demonstrated that phosphorylation may occur in conjunction with Ca^{2+} signaling (Sayeski, et al., 2000; Trinkaus-Randall et al., 2000). The ER is the main storage site for intracellular Ca^{2+}, and mobilization of Ca^{2+} from this store is an essential triggering signal for downstream events, including activation of phosphorylation pathways. Moreover, increases in intracellular calcium concentration ($[Ca^{2+}]_i$) have been associated with membrane fusion for a variety of biological membranes, including some enveloped viruses. For example, binding of HIV-1 gp120 to CD4 and engagement of chemokine coreceptors results in activation of Ca^{2+}-signaling pathways (Davis et al., 1997; Alfano et al., 1999; Liu et al., 2000). Ca^{2+} signaling also may play a role in human cytomegalovirus (HCMV) entry (Keay et al., 1995). Specifically, exposure of human fibroblasts to HCMV mediates an increase in inositol-1,4,5-triphosphate (IP_3), leading to mobilization of internal Ca^{2+} stores. We hypothesize that exposure to HSV might also activate the IP_3 pathway leading to an increase in $[Ca^{2+}]_i$, which, in turn, may activate phosphorylation pathways. Activation of these signaling pathways may facilitate viral penetration and/or the transport of incoming viral capsids to the nucleus.

We tested this hypothesis for HSV-1 and HSV-2 using two different epithelial cell lines. Although the two serotypes are similar in many respects, differences in epidemiology, cell tropism, and spectrum of clinical disease are well known. For example, HSV-1 commonly infects oral mucosa, whereas HSV-2 preferentially infects genital mucosa. With respect to viral entry, serotype differences in the relative contribution of gC and gB toward heparan sulfate binding and in use of gD coreceptors have been described previously (Herold et al., 1991, 1994; Spear et al., 2000). Thus, we explored the role of Ca^{2+}-signaling pathways in viral infection for both serotypes.

Results

Exposure of epithelial cells to HSV induces rapid increase in $[Ca^{2+}]_i$

To evaluate whether HSV infection is associated with activation of Ca^{2+}-signaling pathways, the effects on $[Ca^{2+}]_i$ of exposure of cells to HSV were examined. Vero cells were loaded with Fura-2 and then exposed sequentially to PBS buffer or HSV at a multiplicity of infection (moi) of 5 plaque-forming units (pfu)/cell. Experiments were conducted with wild-type virus or deletion mutants harvested from complementing cells. The complemented viruses behave like wild-type virus with respect to binding and entry (Johnson and Ligas, 1988; Ligas and Johnson, 1988; Roop et al., 1993; Novotny et al., 1996), and have been used in reports of viral entry (Montgomery et al., 1996). An acute increase in superfusate flow rate of PBS alone, simulating the rapid addition of virus to the bathing medium, had no effect on resting $[Ca^{2+}]_i$ (unpublished data). In contrast, exposure to HSV-1 results in a rapid increase in $[Ca^{2+}]_i$, which peaks within one minute, followed by a lower amplitude shoulder and then a return to baseline within 1 min (Fig. 1, bottom left). Similar results were obtained using human cervical epithelial cells (CaSki; Fig. 1, bottom right), although the shoulder was more pronounced and the response persisted for ~3 min before returning to baseline. The mean change (Δ) in peak $[Ca^{2+}]_i$ obtained for HSV-1 on Vero cells, HSV-1 on CaSki cells, or HSV-2 on CaSki cells was 247.5 ± 157 nM, $n = 25$; 354.5 ± 178, $n = 8$; and 443 ± 219, $n = 5$, respectively (not significant; ANOVA; Fig. 2 A).

ER and extracellular Ca^{2+} stores contribute to this viral-induced $[Ca^{2+}]_i$ transient

To determine whether release of ER stores and/or influx of extracellular Ca^{2+} mediate the response to HSV, the effects of pharmacological inhibitors of each on the observed $[Ca^{2+}]_i$ transient were compared. Fura-2–loaded Vero cells were

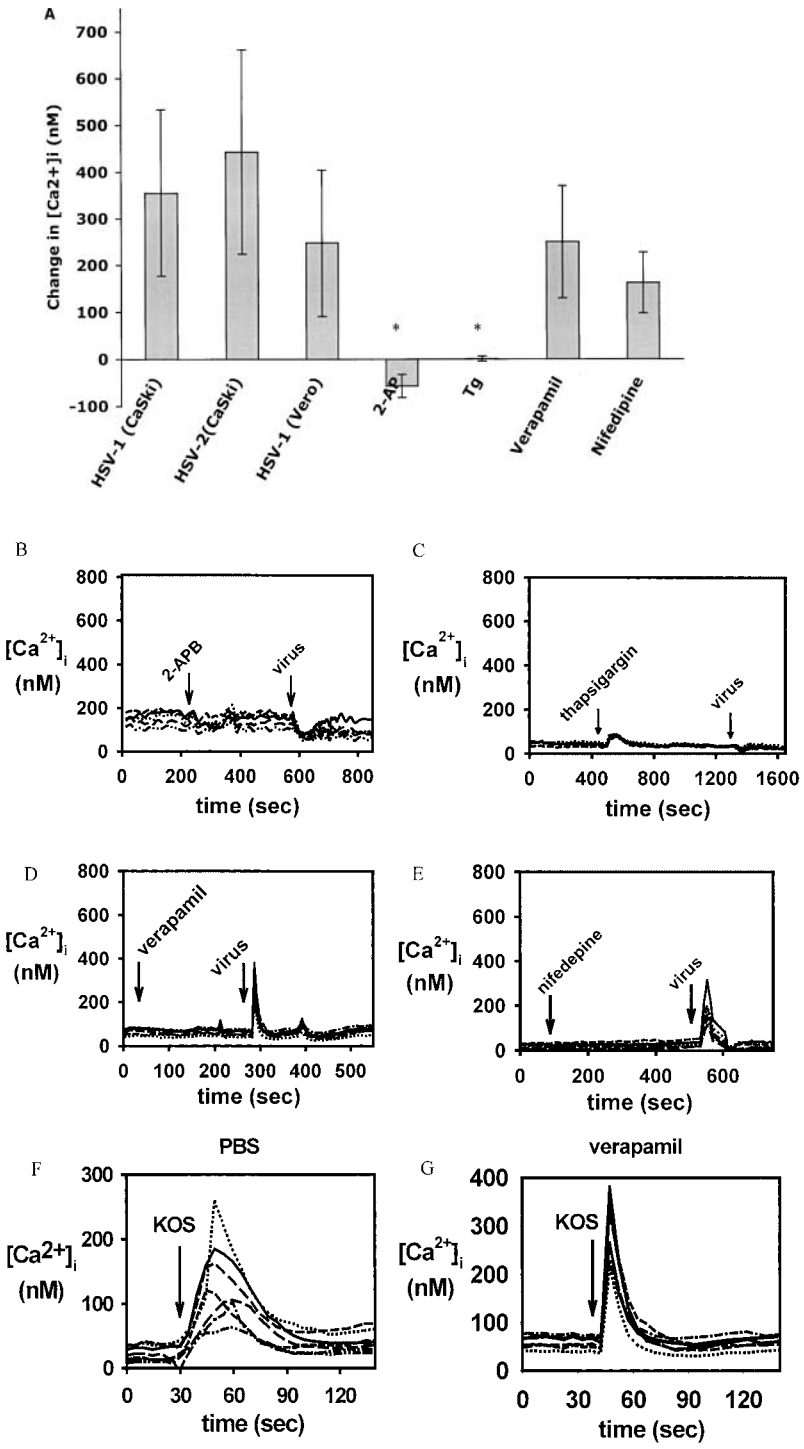

Figure 2. **Changes in $[Ca^{2+}]_i$ in response to HSV-1 or HSV-2 on CaSki cells and in response to HSV-1 in Vero cells pretreated with pharmacological inhibitors.** Means of at least three independent experiments in the absence or presence of each drug are depicted in the bar graph (A); error bars indicate SD and the asterisks denote $P < 0.001$ by ANOVA (see text). Results are shown for individually monitored Vero cells after treatment with 100 μM 2-APB (B), 10 μM Tg (C), 10 μM verapamil (D), or 10 μM nifedipine (E), and subsequent challenge with HSV-1(KOS) (F and G). Time-expanded tracings of cells pretreated with PBS alone or with verapamil are shown in F and G, respectively.

treated with 100 μM 2-aminoethoxydiphenylborate (2-APB) followed by exposure to HSV and $[Ca^{2+}]_i$ response was monitored. 2-APB, an IP_3 receptor antagonist that prevents IP_3-mediated release of ER Ca^{2+}, essentially abolishes the Ca^{2+} response to HSV (Fig. 2 B). The Δ $[Ca^{2+}]_i$ in response to HSV-1 in Vero cells pretreated with 2-APB was -57.1 ± 24.5 nM, $n = 5$ (Fig. 2, A and B; $P < 0.001$, ANOVA). To further examine the importance of ER Ca^{2+} stores to the response, Fura-2–loaded Vero cells were treated with 10 μM thapsigargin (Tg), which induces the release of intracellular ER Ca^{2+} stores and prevents refilling by inhibition of the ER Ca^{2+}-ATPase. Thus, Tg would abrogate the Ca^{2+} response to virus if it requires mobilization of ER stores (Thastrup et al., 1990). Tg induced a modest increase in resting $[Ca^{2+}]_i$ (from 40 ± 11 to 74 ± 13 nM; $n = 10$, $P < 0.01$), which persists for ~3 min in Vero cells before returning to baseline levels. Subsequent exposure to virus results in no further increase in $[Ca^{2+}]_i$ above baseline (Δ $[Ca^{2+}]_i$ 0.89 ± 5.7 nM, $n = 10$; Fig. 2, A and C, $P < 0.001$, ANOVA). Similar results were obtained with CaSki cells, except that Tg induced a greater increase in resting $[Ca^{2+}]_i$ (from 91 ± 35 to 424 ± 119 nM; $n = 6$, $P < 0.01$). Subsequent exposure to virus re-

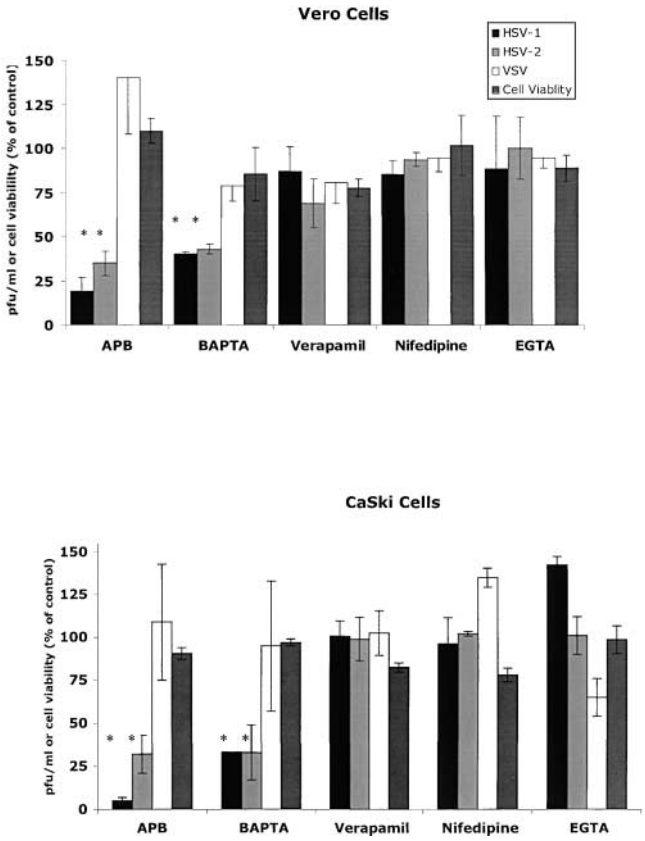

Figure 3. **The ER Ca^{2+} release is required for HSV infection.** The effects of pretreating cells for 1 h with 50 μM BAPTA-AM, 0.5 mM EGTA, or adding 100 μM 2-APB, 10 μM nifedipine, or 10 μM verapamil during viral penetration on HSV-1(KOS), HSV-2(G), or VSV infection of Vero cells (top) or CaSki cells (bottom) were examined. Results are presented as pfu formed in the presence of the indicated concentration of drug as a percentage of pfu formed in cells treated with control buffer (5% DMSO for pharmacological inhibitors or 5% methanol for BAPTA), and are means of at least three independent experiments conducted in duplicate. Cytotoxicity of pharmacological agents was determined in parallel after a 2-h exposure to drug and quantifying viable, proliferating cells at 24 h by MTS assay. Cell viability results are expressed as odu reading in the presence of the drug as a percentage of odu reading in the presence of control buffer, and are means of two independent experiments conducted in triplicate. Asterisks denote $P < 0.001$, ANOVA compared with controls.

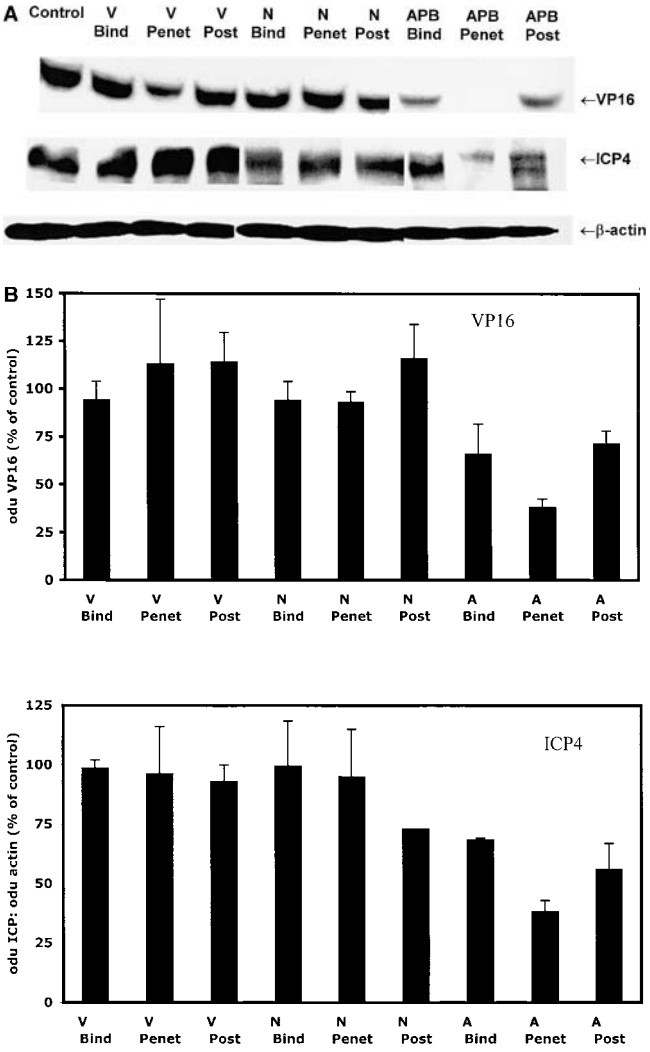

Figure 4. **2-APB prevents VP16 nuclear transport and viral ICP4 expression.** Time-course experiments were conducted to determine the effects of pharmacological inhibitors of Ca^{2+} signaling on VP16 transport to the nucleus and ICP4 expression after infection of CaSki cells with HSV-2(G). 10 μM verapamil (V), 10 μM nifedipine (N), or 100 μM 2-APB (A) were added during the 4°C binding period, at the time of temperature shift (penetration), or immediately after entry. Controls included cells treated with 5% DMSO. Nuclear extracts were prepared 4 h after infection for VP16 (top blot), and cell lysates were prepared 5 h after infection for ICP4 (middle blot). VP16 and ICP4 were detected by analyzing Western blots; lanes were loaded with extracts or lysates from equivalent cell numbers. The blots of cell lysates were also probed with mAb for β-actin to control for loading (bottom blot). The blots were scanned and the background from mock-infected cells was subtracted. (B) The odu for viral protein per odu for β-actin as a percentage of controls. Results are the mean ± SD obtained from three independent experiments.

sulted in a minimal increase in $[Ca^{2+}]_i$ above baseline ($\Delta [Ca^{2+}]_i$ 22 ± 13 nM, $n = 6$, $P < 0.001$, ANOVA).

To explore the possibility that Ca^{2+} influx across voltage-operated channels also contributes to the response to HSV, cells were pretreated with 10-μM concentrations of verapamil or nifedipine. Minimal change in the amplitude of $[Ca^{2+}]_i$ response to virus was observed. The $\Delta [Ca^{2+}]_i$ in response to HSV-1 in nifedipine- or verapamil-treated Vero cells was 163 ± 65 ($n = 11$) and 251 ± 120 ($n = 12$), respectively (Fig. 2, A, D, and E). However, the response to virus was modified in cells treated with these agents as reflected in the loss of the shoulder (Fig. 2, F and G). Similar results were obtained for HSV-2 and with CaSki cells (unpublished data). Together, these results suggest that the peak response to virus reflects release of ER Ca^{2+} stores, whereas the shoulder may represent influx of Ca^{2+} across voltage-operated channels. The results obtained are consistent with the notion that plasma membrane Ca^{2+} channels may be linked to the IP_3 receptors. Similar results have been described in several other systems (Berridge, 1995; Parekh and Penner, 1997; Uhlen et al., 2000; Bakowski et al., 2001; Straube and Parekh, 2001; Valencia et al., 2001).

Effects of Ca^{2+} inhibitors on viral infection

To determine whether the Ca^{2+} response is important for viral infection, synchronized infectivity assays were conducted

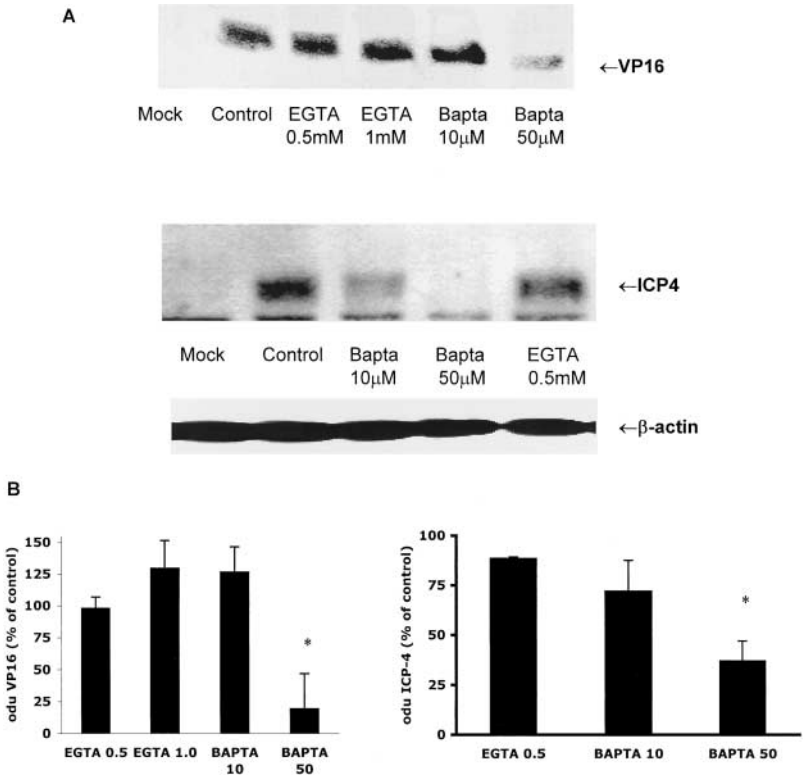

Figure 5. **Effects of Ca^{2+} chelators on VP16 transport and ICP4 expression.** CaSki cells were pretreated with BAPTA-AM, EGTA, or 5% methanol in PBS (control) for 2 h, washed three times, and then exposed to HSV-2(G) and nuclear extracts or cell lysates prepared as described in Materials and methods. (A) Representative blots probed for VP16 (top blot) or ICP4 (middle blot). Blots were also probed with mAb for β-actin to control for loading (bottom blot). The blots were scanned and the background from mock-infected cells was subtracted. (B) The odu for viral protein per odu for β-actin as a percentage of control (HSV-2 in the absence of pharmacological inhibitor). Results are the mean ± SD obtained from three independent experiments; asterisks indicate $P < 0.001$ by ANOVA.

in which 100 μM 2-APB, 10 μM nifedipine, or 10 μM verapamil were added at the time of viral penetration for 1 h. HSV attachment, which occurs at 4°C, can be differentiated from penetration, which occurs after a shift to 37°C. The effects of these pharmacological agents on cell viability were determined in parallel. Although Tg does not significantly reduce cell viability at a concentration of 10 μM, it is known to have pleiotropic effects on cell function, including inhibition of protein synthesis at concentrations as low as 30 nM (Soboloff and Berger, 2002), and for this reason, Tg was not included. Results for Vero and CaSki cells are summarized in Fig. 3. HSV-1 and HSV-2 but not vesicular stomatitis virus (VSV) infections were reduced after treatment of cells with 2-APB ($P < 0.001$, ANOVA). In contrast, the Ca^{2+} channel blockers had little or no effect on viral infection (Fig. 3). These results suggest that release of IP_3-sensitive ER stores is required for HSV infection. To further assess the importance of $[Ca^{2+}]_i$ on HSV infection, the cells were pretreated for 2 h with 50 μM BAPTA-AM, a cell-permeable cytosolic Ca^{2+} chelator, or with 0.5 mM EGTA, a concentration that chelates extracellular Ca^{2+} and thereby prevents entrance of extracellular Ca^{2+} (Zwick et al., 1999, Bouchard et al., 2001), and were then infected with virus. Cell viability was monitored in parallel. Pretreatment of cells with BAPTA-AM (but not EGTA) reduced HSV infection to a similar extent as that observed with 2-APB (Fig. 3, $P < 0.001$, ANOVA).

To more specifically examine which steps in HSV infection require activation of the IP_3 pathway, additional experiments were conducted and infection was monitored by examining transport of VP16, a viral tegument protein. Detection of VP16 in the nucleus is one of the earliest markers of successful viral entry and transport. Synchronized infectivity assays were conducted and the pharmacological agents were added during binding (at 4°C for 2 h), penetration (at the time of temperature shift for 15 min), or for 1 h immediately after penetration (post-citrate treatment). Controls included cells treated with 5% DMSO. Nuclear extracts were prepared 4 h after infection and the nuclear transport of VP16 was compared using Western blots. In parallel, the expression of the viral immediate early gene product, ICP4, was compared by preparing Western blots of cell lysates 5 h after infection. The VP16 and ICP4 blots were scanned, the background from mock infected lanes subtracted, and the optical densitometry corrected for differences in loading (optical densitometry units [odu] for viral protein per odu for β-actin). A representative blot for VP16 after infection of CaSki cells with HSV-2(G) is shown in Fig. 4 A (top blot). VP16 transport to the nucleus is inhibited if cells are treated with 2-APB, but not nifedipine or verapamil. The inhibition is greatest when 2-APB is added during penetration. Similarly, 2-APB, but not nifedipine or verapamil, also reduces ICP4 expression (Fig. 4 A, middle blot). Results from three independent experiments are summarized graphically in Fig. 4 B. Similar results were obtained using Vero cells and HSV-1 (unpublished data). These results suggest that IP_3-mediated Ca^{2+} signaling is required during penetration, and either facilitates the fusion process and/or transport of incoming capsids and tegument proteins to the nucleus.

Similar experiments were conducted using BAPTA-AM and EGTA. Cells were pretreated for 2 h with the chelators, infected with virus, and nuclear extracts or cell lysates were prepared at 4 and 5 h after infection, respectively. Representative gels with CaSki cells and HSV-2 are shown in Fig. 5 A. Consistent with the results obtained with the pharmaco-

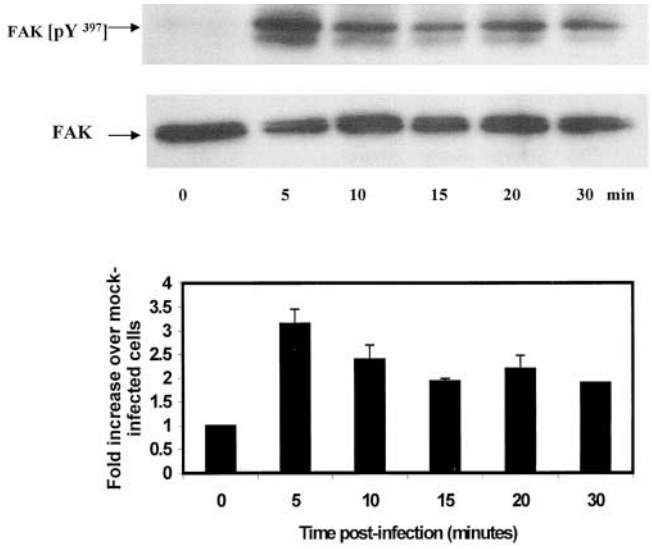

Figure 6. **HSV induces FAK phosphorylation within 5 min of exposure to virus.** CaSki cells were serum starved overnight, exposed to HSV-2(G) or mock infected, and at the indicated times after infection, cell lysates were prepared and proteins were separated, transferred by Western blotting, and incubated with anti-FAK pY^{397} antibody. Blots were then stripped and reprobed with mAb to total FAK. Blots were scanned and the results are expressed as the increase in levels of phosphorylated FAK as a percentage of total FAK compared with mock-infected cells. Results are the mean ± SD obtained from three independent experiments.

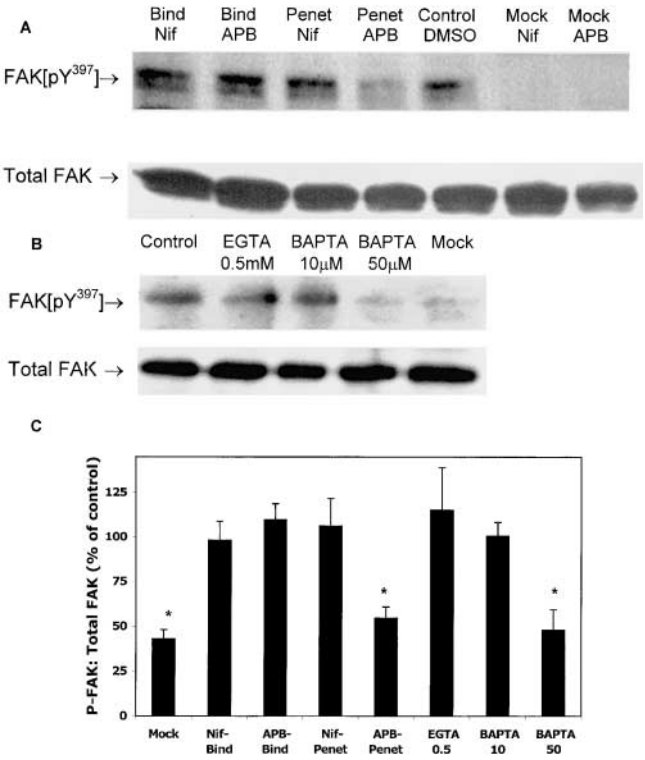

Figure 7. **2-APB and BAPTA-AM prevent viral-induced FAK phosphorylation.** CaSki cells were serum starved overnight, and a synchronized infection with HSV-2(G) was conducted as described in Materials and methods. 100 μM 2-APB, 10 μM nifedipine, or control buffer (5% DMSO) was added to cells at the time of binding or penetration (temperature shift, 15 min, 37°C). Cells were treated with low pH citrate buffer, washed, and cell lysates were prepared 10 min after citrate treatment. Western blots were prepared, probed with anti-FAK pY^{397}, and scanned by optical densitometry. Blots were then stripped and reprobed with mAb to total FAK. A representative blot is shown in A. Alternatively, cells were pretreated for 2 h with BAPTA-AM, EGTA, or 5% methanol in PBS (control), and then exposed to HSV-2(G). (B) Lysates were prepared 5 min after infection and analyzed by Western blotting for FAK phosphorylation. (C) Results obtained from three independent experiments are summarized graphically as odu of phosphorylated FAK:odu total FAK as a percentage of control (HSV-2(G) in the absence of any inhibitors). Results are the mean ± SD obtained from three independent experiments; asterisks indicate $P < 0.001$, ANOVA.

logical inhibitors, pretreatment of cells with the intracellular (but not the extracellular) chelator reduced transport of VP16 to the nucleus and expression of ICP4 by ~60–70% (Fig. 5 B; $P < 0.001$, ANOVA). Similar results were obtained with Vero cells and HSV-1. The inhibitory effects of BAPTA-AM were concentration dependent, presumably reflecting low esterase activity in CaSki cells (hydrolysis of the ester group by cellular esterases is required for chelation).

Inhibition of Ca^{2+} signaling also prevents phosphorylation of FAK

HSV entry is associated with tyrosine phosphorylation of several cellular proteins within 5–10 min after infection (Qie et al., 1999). We hypothesized that one of these proteins may be FAK and that activation of FAK phosphorylation pathways may be linked to the release of IP_3-sensitive ER Ca^{2+} stores. To explore this possibility, cells were serum starved overnight and mock infected or exposed to HSV at 37°C. At various times after exposure, cell lysates were prepared and examined for expression of phosphorylated and total FAK by Western blotting. As shown in Fig. 6, HSV-2 induces a three- to fourfold increase in FAK phosphorylation within 5 min after exposure. Similar results were obtained for Vero cells. To determine if the release of ER Ca^{2+} is linked to the observed phosphorylation of FAK, synchronized infection experiments were conducted in which 2-APB or nifedipine were added during viral binding or penetration. FAK phosphorylation was examined 10 min after citrate treatment (Fig. 7 A). Alternatively, cells were pretreated with BAPTA-AM or EGTA for 2 h and then challenged with virus (Fig. 7 B). Results from three independent experiments are summarized in the graph (Fig. 7 C). Pretreatment with 50 μM BAPTA-AM and addition of 2-APB during viral penetration reduced the FAK phosphorylation (expressed as percentage of total FAK) to levels similar to that observed for mock-infected cells. In contrast, EGTA and nifedipine had little or no effect. These findings suggest that FAK phosphorylation may be linked to IP_3 pathway activation and release of internal $[Ca^{2+}]_i$ stores. Alternatively, it may be that viral entry itself, and not the release of internal $[Ca^{2+}]_i$, promotes FAK phosphorylation.

Viral binding is not sufficient to induce Ca^{2+} signaling or FAK phosphorylation

For both HIV and HCMV, binding of viral glycoproteins to the cell surface is sufficient to induce a Ca^{2+} response (Keay et al., 1995; Keay and Baldwin, 1996; Alfano et al., 1999; Liu et al., 2000). To determine if this is also the case for

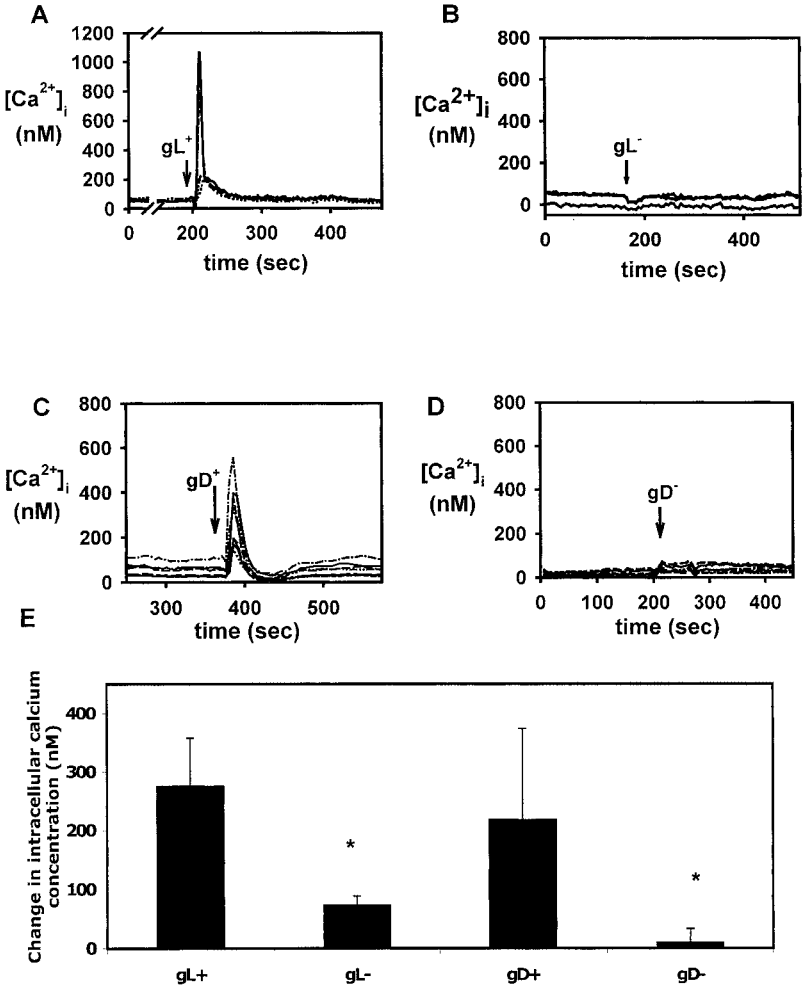

Figure 8. **Viruses deleted in gL or gD fail to induce a Ca^{2+} response.** Fura-loaded Vero cells were exposed to gL86 (A and B) or KOSgDβ (C and D) purified from complementing (left) or noncomplementing (right) Vero cells, and the $[Ca^{2+}]_i$ (nM) response was monitored. (E) Results from a single experiment in which 3–7 cells were monitored are shown, and the mean Δ $[Ca^{2+}]_i$ obtained from two independent experiments is depicted graphically. Results are the mean ± SD obtained from three independent experiments; asterisks indicate $P < 0.001$, ANOVA.

HSV, the effect of exposing cells to equivalent particle numbers of HSV-1(KOS) viruses deleted in gL or gD purified from noncomplementing Vero cells (phenotypically deleted) was compared with results obtained with virus purified from complementing cells (phenotypically wild-type). Deletion of gD or gL results in virions that bind, but fail to penetrate (Ligas and Johnson, 1988; Roop et al., 1993). The relative concentration of purified viral particles was determined by comparing the odu of the gB or gD band after SDS-PAGE and Western blotting, as described previously (Qie et al., 1999; Cheshenko and Herold, 2002). As shown in Fig. 8, there was little increase in $[Ca^{2+}]_i$ after exposure of cells to noncomplemented gD- or gL-deleted viruses (Δ $[Ca^{2+}]_i$ = 9.6 ± 23.6, n = 11 and 73 ± 16, n = 5, respectively). The Ca^{2+} response was restored when cells were exposed to the complemented virus (Δ $[Ca^{2+}]_i$ = 218 ± 156, n = 11 and 276 ± 82, n = 5, respectively. These results suggests that binding in the absence of either gD or gL is not sufficient to induce changes in $[Ca^{2+}]_i$. An HSV-2(G) virus deleted in gB-2, which is impaired both in binding and penetration, also failed to induce a Ca^{2+} response (unpublished data).

To determine whether viral penetration is required to induce FAK phosphorylation, the effects of exposing serum-starved cells to equivalent particle numbers of the deletion viruses purified from complementing or noncomplementing cells or heat-inactivated (HI) virus were compared. Previous reports have shown that treatment of virus for 1 h at 56°C is similar to ultraviolet light inactivation with respect to entry (Moriuchi et al., 2000). Moreover, in pilot experiments, we found that HI virus enters as indicated by successful transport of VP16 to the nucleus, but fails to express ICP4 (unpublished data). Cells were exposed to each of the purified viral preparations and Western blots for phosphorylated FAK and total FAK were prepared 10 min post exposure. Representative gels are shown in Fig. 9. There was no increase in FAK phosphorylation in cells exposed to virus deleted in gL, gD, or gB compared with mock-infected cells. In contrast, a twofold increase in FAK phosphorylation was observed if cells were exposed to virus purified from complementing cell lines or to HI virus. Together, these results suggest that the full complement of glycoproteins required for penetration is required to induce signaling events.

Discussion

We have shown that release of IP_3-sensitive Ca^{2+} stores held within the ER facilitates HSV-1 and HSV-2 entry and/or transport of incoming viral capsids to the nucleus. A possible paradigm is depicted in Fig. 10. After interactions between heparan sulfate proteoglycans and gC (HSV-1; Herold et al., 1991) or gB (HSV-2; Cheshenko and Herold, 2002) and engagement of herpes virus entry mediator coreceptors by

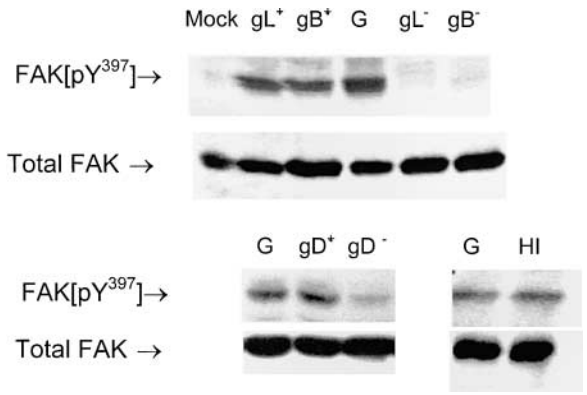

Figure 9. **Viral penetration is required for FAK phosphorylation.** Serum-starved CaSki cells were mock infected or exposed to HSV-1(KOS)gL86, HSV-1(KOS) gDβ, or HSV-2(g)gB-2$^-$ grown on complementing (+) or noncomplementing (−) cells as indicated or exposed to HSV-2 (G) or HI-HSV-2 (HI) virus and FAK phosphorylation monitored by preparing cell lysates 10 min after citrate treatment. Blots were probed with anti-FAK pY^{397} and then stripped and reprobed with mAb to total FAK. Gels are representative of at least two independent experiments.

gD (Spear et al., 2000), we propose that a signal is transduced, which may activate phosphoinositide-specific PLCγ. PLCγ catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3. IP_3 diffuses through the cytosol, binds to an IP_3-sensitive receptor, and opens Ca^{2+} channels in the membrane of the ER, releasing Ca^{2+} into the cytosol. The release of ER Ca^{2+} stores, in turn, may promote opening of Ca^{2+} channels at the plasma membrane, as suggested by the shoulder in the Ca^{2+} response and the finding that pretreatment with nifedipine and verapamil results in a loss of this shoulder. Release of IP_3-sensitive stores, but not the influx of Ca^{2+} across the plasma membrane, is important for HSV infection as indicated by the inhibitory effects of 2-APB (but not verapamil and nifedipine) on transport of VP16 to the nucleus, expression of ICP4, and plaque formation. This is further supported by the finding that chelation of intracellular Ca^{2+} with BAPTA-AM prevents VP16 transport and ICP4 expression. Possibly, the increase in $[Ca^{2+}]_i$ facilitates membrane fusion between the HSV envelope and cell plasma membrane. Alternatively, the signaling pathway may be important for delivery of viral capsids to the cytoplasm or to their nuclear transport.

HSV invasion also triggers phosphorylation of FAK. This response occurs within 5–10 min after exposure to virus and could be triggered directly by the increases in $[Ca^{2+}]_i$ or in response to viral entry. The notion that FAK phosphorylation is linked to the Ca^{2+} response is supported by the finding that treatment of cells with 2-APB or BAPTA-AM reduced subsequent FAK phosphorylation. Phosphorylation of FAK promotes reorganization of the actin cytoskeleton, a process that may be important in nuclear trafficking of internalized virus. Microtubules have been shown to play a role in transport of the HSV viral capsid to the cell nucleus, although the role of FAK was not evaluated (Sodeik et al., 1997). Notably, phosphorylation of FAK and related cytoskeletal proteins has also been shown to be important in HIV and adenoviral invasion. Binding of HIV gp120 to CD4 and chemokine coreceptors induces phosphorylation and intracellular association of FAK and CCR5 (Cicala et al., 1999). Adenoviral internalization is associated with phosphorylation of FAK and p130cas (Li et al., 2000).

Our analyses suggest that binding of virus to heparan sulfate proteoglycan receptors and engagement of gD coreceptors are not sufficient to trigger the cellular signaling pathways. Similarly, interactions between gH–gL and the cell, in the absence of gD or gB, is not adequate. This follows from the observations that viruses deleted in gB, gD, or gL fail to induce the signaling events. In contrast, HI virus, which retains the ability to enter cells, induces FAK phosphorylation. The requirement for the full complement of essential glycoproteins (gB, gD, and gH–gL) to trigger the signaling events suggests that the pathway is activated in response to initiation of the fusion process or subsequent early events in invasion. These results differ from those obtained with the related β-herpes virus, HCMV. Activation of a cellular receptor for HCMV gH–gL (a 92.5-kD cellular protein) directly or indirectly by anti-idiotypic antibodies mediates transmembrane signaling events resulting in increases in $[Ca^{2+}]_i$ (Keay et al., 1995; Milne et al., 1998; Baldwin et al., 2000). No cellular

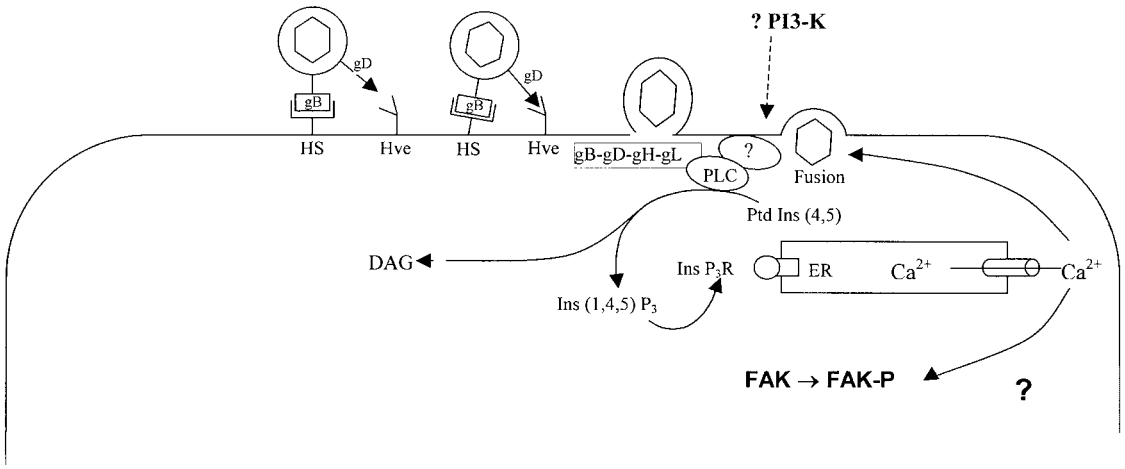

Figure 10. **Model of HSV entry.** See Discussion for description.

receptor for the gH–gL complex of HSV-1 or HSV-2 has yet been identified. Possibly, the gH–gL complex for HSV (in the presence of gB and gD) also engages a cellular receptor that directly or indirectly activates PLCγ. Recent experiments have shown that phosphoinositide-specific PLCγ may be activated by phosphatidylinositol-3-kinase, which can act upstream of PLCγ (Bierne et al., 2000).

In summary, these analyses demonstrate that Ca^{2+} signaling pathways play a key role in facilitating early events in HSV-1 and HSV-2 invasion. Because the signaling responses occur within minutes after exposure to virus, it seems likely that they are associated with fusion of the viral envelope and cell membrane and/or an immediate post-entry event. Activation and release of IP_3-sensitive Ca^{2+} stores and subsequent phosphorylation of FAK are dependent on the presence of the full complement of essential envelope glycoproteins. Defining the specific cellular pathways activated during HSV entry and precisely how the virus triggers these pathways is key to understanding the process of viral invasion, and should facilitate development of novel strategies to prevent HSV entry and infection.

Materials and methods

Cells and viruses

Vero (monkey kidney epithelial) and CaSki (human cervical epithelial) cells were obtained from the American Type Culture Collection and passed in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS. The cell lines 79VB4 and VD60 (provided by P. Spear, Northwestern University, Evanston, IL) and VgB2 are gL-, gD-, and gB-2–expressing Vero cell lines, respectively. These cell lines were passed in DMEM supplemented with 10% FBS. The 79VB4 cells were grown in the presence of 200 μg/ml G418 sulfate (Montgomery et al., 1996) and the VgB2 cell line in the presence of 500 μg/ml G418 sulfate (Cheshenko and Herold, 2002). The wild-type viruses HSV-1(KOS) (provided by P. Spear), HSV-2(G) (provided by B. Roizman, University of Chicago, Chicago, IL), and VSV-Indiana (provided by P. Palese, Mount Sinai School of Medicine, New York, NY) were grown on Vero cells. HI virus was prepared by heating to 56°C for 1 h (Moriuchi et al., 2000). HSV-1 (KOS)(gL86), a mutant in which the *Escherichia coli* β-galactosidase gene replaces part of the gL ORF, was grown on 79VB4 cells or Vero cells as indicated (Montgomery et al., 1996; Qie et al., 1999). HSV-1(KOS)gDβ, a mutant in which the *E. coli* β-galactosidase gene replaces part of the gD ORF, was grown on VD60 or Vero cells as indicated (Dean et al., 1994). HSV-2(G)gB-2^{-}, a mutant in which the hygromycin/enhanced green fluorescent fusion protein replaces part of the gB-2 ORF, was grown on VgB-2 or Vero cells as indicated (Cheshenko and Herold, 2002).

Purification and quantification of viruses

Viruses were purified from Vero or complementing cells on dextran gradients as described previously (Herold et al., 1991, 1994). Titers of the purified virus were determined by plaque assays. Relative viral particle numbers were determined by comparing the amounts of gB or gD by optical densitometry after Western blotting with mAb 1123 (anti-gB) or mAb1103 (anti-gD) (Goodwin Institute, Plantation, FL), as described previously (Tal-Singer et al., 1995; Qie et al., 1999; Cheshenko and Herold, 2002).

Reagents

Verapamil, nifedipine, 2-APB, Tg, EGTA, EGTA-AM, and ionomycin were purchased from Calbiochem and diluted in DMSO or PBS per manufacturer's instructions. The acetoxymethyl ester of Fura-2 (Fura-2/AM) and the cell-permeable cytosolic Ca^{2+} chelator, BAPTA-AM, were purchased from Molecular Probes, Inc. Heparin was purchased from Sigma-Aldrich.

Measurement of $[Ca^{2+}]_i$

Cells were loaded with 25 μM Fura-2/AM prepared in PBS for 30–60 min, rinsed with PBS for 30 min at 37°C, and then exposed sequentially to buffer or pharmacological inhibitors and purified HSV diluted in PBS at 37°C. The viral inoculum was equivalent to an moi equal to 1–5 pfu/cell (based on titer on complementing cells) or an equivalent number of viral particles for noninfectious virus. Using an inverted epifluorescence microscope (Eclipse TE300; Nikon) linked to a cooled CCD camera (Pentamax; Princeton Instruments) interfaced with a digital imaging system (MetaFluor; Universal Imaging Corp.), $[Ca^{2+}]_i$ was measured in individually identified Fura-2–loaded cells visualized using an S Fluor 40× objective (NA 0.9, WD 0.3; Nikon) as described previously (Woda et al., 2002). Cells were alternately excited at 340 and 380 nm, and the images were digitized for subsequent analysis. Images were acquired every 2–10 s. An intracellular calibration was performed at the conclusion of each experiment according to previously described techniques (Grynkiewicz et al., 1985; Woda et al., 2002). The 340/380-nm fluorescence ratio was determined initially in the presence of a Ca^{2+}-free bath plus 10 μM EGTA-AM (R_{min}), and then in a 2-mM Ca^{2+} bath containing 10 μM ionomycin (R_{max}). The equation used to calculate experimental values of $[Ca^{2+}]_i$ was: $[Kd(R-R_{min})/R_{max}-R)](S_{f2}/S_{b2})$, where R is the observed ratio of emitted light, Kd is the dissociation constant for Fura-2 and Ca^{2+} (assumed to be 224 nM), and S_{f2} and S_{b2} are the fluorescence signals of free and bound dye at 380 nm, respectively (Grynkiewicz et al., 1985). 7–15 cells were monitored for each experiment.

Plaque assays

Synchronized plaque assays in which pharmacological inhibitors were added during viral penetration were conducted as described previously (Herold et al., 2002). In brief, cells in 6-well dishes were precooled and exposed to HSV or VSV at 4°C for 2 h to allow binding. The moi was selected to yield ~200–500 pfu/well (e.g., moi ~0.005 pfu/cell) on control wells. Unbound virus was removed and the cells were washed three times with PBS and then treated with 10 μM verapamil or nifedipine, 100 μM 2-APB, 10 μM Tg, or buffer (DMSO or PBS). Cells were immediately transferred to 37°C to allow viral penetration for 1 h. Unpenetrated virus was inactivated by washing the cell monolayer with a low pH citrate buffer (50 mM sodium citrate and 4 mM KCl, adjusted to pH 3.0) for 2 min, and then by washing three times with PBS. The cells were then overlaid with medium containing 0.5% methylcellulose for 48 h. For HSV-1(KOS) and HSV-2(G), plaques were counted by immunoassay (black-plaque; Herold et al., 1991). For VSV, cells were overlaid with 0.1% methylcellulose, fixed after 24 h with methanol, and stained with Giemsa.

VP16 and ICP4 time-course assays

To examine which steps in HSV infection are inhibited by the pharmacological agents, the time of introduction of the drug was varied and infection was monitored by examining transport of the tegument protein VP16 to the nucleus or expression of immediate early gene product, ICP4. Synchronized infectivity assays were conducted as described above, except the moi was equivalent to 1 pfu/cell and the penetration time (time from temperature shift to citrate treatment) was reduced to 15 min. The pharmacological agents or control buffers were added during the 4°C binding period, at the time the cells were transferred to 37°C (penetration), or immediately after the citrate treatment for 1 h. The cells were then overlaid with medium and viral infection was monitored by examining transport of VP16 or expression of ICP4 as described below.

To examine transport of VP16 to the nucleus, nuclear extracts were prepared 4 h after infection as described previously (Melchjorsen et al., 2002). The nuclear proteins were separated on an 8.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (PerkinElmer) using a Trans-Blot system (Bio-Rad Laboratories) and blocked overnight in 5% milk-TBS. Membranes were incubated with mouse anti-VP16 (1:500; Santa Cruz Biotechnology, Inc.), diluted in 5% milk-TBS for 2 h, and rinsed extensively in TBST (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20). The membranes were then incubated with HRP-conjugated goat anti–mouse IgG (1:1,000; Calbiochem) in 5% milk-PBS for 2 h. After rinsing, the membranes were developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). Blots were scanned and analyzed using the Gel Doc 2000 system (Bio-Rad Laboratories).

To examine ICP4 expression, infected cells were harvested 5 h after infection in lysis buffer (1% SDS, 50 mM Tris-Cl, pH 7.4, 55 mM EDTA, and I mM DTT), which was supplemented with complete protease inhibitors (Roche). The supernatant (soluble fraction) and pellet (insoluble fraction) were separated by centrifugation at 16,000 *g* at 4°C, and the protein concentrations in the supernatants were determined using the DC Protein Assay (Bio-Rad Laboratories). The supernatants were suspended in SDS sample buffer (100 mM Tris-Cl, pH 6.8, 1% β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) boiled for 4 min. The proteins were separated on an 8.5% SDS-polyacrylamide gel and transferred to a PVDF membrane (PerkinElmer) using a Trans-Blot system (Bio-Rad Laboratories) and blocked overnight in 5% milk-TBS. Membranes were incubated

with mouse anti-ICP4 1101 (Goodwin Institute, Plantation, FL) or anti-β-actin A5441 (Sigma-Aldrich) diluted in 5% milk-TBS for 2 h, then rinsed extensively in TBST (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20). The membranes were then incubated with HRP-conjugated goat anti–mouse IgG (1:1,000; Calbiochem) in 5% milk-PBS for 2 h. After rinsing, the membranes were developed using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). Blots were scanned and analyzed using the Gel Doc 2000 system.

Detection of FAK phosphorylation by immunoblot

Nearly confluent CaSki or Vero monolayers were preincubated with serum-free medium for 24 h before infection, and were then exposed to HSV viruses at an moi of 10 pfu/cell for 5, 10, 20, or 30 min. Cell lysates were prepared as described above. Proteins were separated by SDS-PAGE and immunoblots prepared as described above. Membranes were incubated with a 1:1,000 dilution of rabbit anti-FAK[pY^{397}], which recognizes the autophosphorylation site of FAK (Biosource International). The membranes were then stripped and reincubated with a 1:1,000 dilution of mouse anti-FAK mAb (F-15020; Transduction Laboratories), which recognizes total FAK.

Effects of BAPTA-AM or EGTA on viral infection and FAK phosphorylation

CaSki or Vero cells were pretreated for 2 h at 37°C with 10 or 50 μM BAPTA-AM or with 0.5 mM EGTA, washed three times, and exposed to HSV or VSV as described above. Infection was monitored using plaque assays, transport of VP16 to the nucleus, or expression of ICP4. To examine the effects of the chelators on FAK phosphorylation, cells were serum starved for 24 h, treated with the chelators for 2 h, and then infected. At various times after infection, cell lysates were prepared as described above. Proteins were separated by SDS-PAGE and immunoblots were incubated with antibodies directed against FAK[pY^{397}] or ICP4.

Cell viability assay

The effects of the pharmacological agents on cell viability were determined by the metabolic reduction of the tetrazolium salt MTS (CellTiter 96®; Promega). Half-confluent Vero or CaSki cells were exposed to various concentrations of each drug for the indicated times, washed three times, and overlaid with fresh medium. Cell viability was examined at 24 h. Controls included cells exposed to media in the absence of any drugs and cells exposed to 0.1% nonoxynol-9 (Sigma-Aldrich), a detergent cytotoxic to cells in culture (Herold et al., 1999).

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