Capsaicin-induced reactivation of latent herpes simplex virus type 1 in sensory neurons in culture

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Herpes simplex virus type 1 (HSV-1) produces a lifelong latent infection in neurons of the peripheral nervous system, primarily in the trigeminal and dorsal root ganglia. Neurons of these ganglia express high levels of the capsaicin receptor, also known as the vanilloid receptor-1 (VR-1). VR-1 is a non-selective ion channel, found on sensory neurons, that primarily fluxes Ca^{2+} ions in response to various stimuli, including physiologically acidic conditions, heat greater than 45°C and noxious compounds such as capsaicin. Using an in vitro neuronal model to study HSV-1 latency and reactivation, we found that agonists of the VR-1 channel – capsaicin and heat – resulted in reactivation of latent HSV-1. Capsaicin-induced reactivation of HSV-1 latently infected neurons was dose-dependent. Additionally, activation of VR-1 at its optimal temperature of 46°C caused a significant increase in virus titre, which could be attenuated with the VR-1 antagonist, capsazepine. VR-1 activation that resulted in HSV-1 reactivation was calcium-dependent, since the calcium chelator BAPTA significantly reduced reactivation following treatment with capsaicin and forskolin. Taken together, these results suggest that activation of the VR-1 channel, often associated with increases in intracellular calcium, results in HSV-1 reactivation in sensory neurons.

INTRODUCTION

The most common clinical manifestation of a herpes simplex virus type 1 (HSV-1) infection is fever blisters on the epithelial surface. Although generally not life-threatening, HSV-1 can have devastating effects on infections of the eye or the central nervous system. HSV-1 is the leading cause of both blindness associated with a viral infection and sporadic fatal encephalitis (Cook, 1992). The initial site of HSV-1 infection is generally the dermal epithelium, where the virus replicates efficiently resulting in lesions or fever blisters. During the primary infection, HSV-1 gains access to peripheral sensory nerve termini, where virus uptake and retrograde transport to the neuronal nucleus occur. Once HSV-1 infects the peripheral nervous system, it can produce a lifelong latent infection. The presence of viral DNA as an episome in the nucleus and the absence of infectious virus commonly define a latent infection (Baringer & Swoveland, 1973; Wagner & Bloom, 1997). Additionally, an RNA transcript called the latency-associated transcript (LAT) is often detected during the latent infection (Stevens et al., 1987). In humans, diverse stimuli can cause virus reactivation, including fever, prolonged UV exposure or sunburn, severe burns, menstrual cycle and stress (Epstein, 1941; Hill et al., 1977). Upon reactivation, new viral progeny are produced and these new infectious viral particles are anterogradely transported back to the dermal epithelium to produce lesions and transmissible virus. The mechanism controlling HSV-1 reactivation remains poorly understood to date.

In HSV-1 latently infected individuals, the observed reactivation following fever or severe burns suggests that noxious temperatures may induce a signalling mechanism leading to reactivation of latent virus. The receptor in the peripheral nervous system that responds to noxious heat stimulus is the capsaicin receptor, also known as the vanilloid receptor-1 (VR-1). VR-1 is an ion channel that preferentially fluxes calcium ions on activation and is primarily, but not exclusively, found on nociceptor neurons (Caterina et al., 2000; Mezey et al., 2000). Nociceptors or pain-perceiving neurons exist at the dermal epithelial layer as free nerve endings, in contrast to other peripheral neurons that contain laminates of some form protecting the nerve endings in the tissue. Because the nociceptor is the only free nerve ending in the dermal epithelium, it may be more susceptible to virus uptake and more likely to harbour latent virus. The exposed free nerve terminal of the nociceptor responds primarily to painful stimuli including extreme heat, painful mechanical stimulus and noxious chemicals. Nociceptor neurons are not only sensitive to increases in temperature that exceed 45°C but are also activated by the pungent extract from hot chilli peppers, capsaicin (Schmelz et al., 2000). These stimuli, which activate the nociceptor, also cause HSV-1 reactivation.
To elucidate the signalling pathway involved in fever-induced HSV-1 reactivation, we examined the effects of heat-shock and capsaicin in an in vitro neuronal model of HSV-1 latency. This model allows the direct investigation of the signal transduction pathway specific for HSV-1 reactivation in sensory neurons in a defined and controlled environment (Wilcox & Johnson, 1987). Through immunocytochemistry, we found that the majority of neurons in the cultures were VR-1-positive. Our results, both with heat-shock and capsaicin treatment, indicated that VR-1 activation leads to reactivation of latent HSV-1 in sensory neurons. Additionally, reactivation through VR-1 activation could be attenuated with an antagonist to VR-1 or the calcium chelator, BAPTA. These results suggest that elevation of intracellular calcium induced by VR-1 activation regulates HSV-1 reactivation in neurons.

METHODS

Cell culture. Vero cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% foetal bovine serum (Invitrogen). Sensory neuron cultures were prepared from dorsal root ganglia (DRG) of embryonic day 15 Sprague–Dawley rats, as previously described (Smith & Wilcox, 1996; Wilcox et al., 1990). Neurons were plated on to 24-well plates at a cell density of approximately 1–5 × 10^4 cells per well. DMEM/F12 medium supplemented with 10% newborn bovine serum and 100 ng/mL acycloguanosine (Sigma) 24 h prior to inoculation with HSV-1 and for the following 7 days after inoculation. Neuronal cultures were infected with approximately 10 pfu HSV-1 (17+) per neuron (m.o.i. = 10). The cells were incubated with the virus for 1 h and subsequently washed to remove any virus that did not absorb during the incubation period. In the presence of acycloguanosine, HSV-1 establishes a latent infection and can be detected as an episome in the nucleus (Wilcox et al., 1997). Furthermore, we have previously observed the presence of LAT in the HSV-1 latently infected DRG cultures (Doerig et al., 1991).

Establishment of latent HSV-1 infections. Latent HSV-1 infections in neuronal cultures were established as previously described (Smith & Wilcox, 1996; Wilcox & Johnson, 1988; Wilcox et al., 1990). Briefly, after the neurons had been allowed to mature in tissue culture for 21 days, they were treated with 50 μM acycloguanosine (Sigma) 24 h prior to inoculation with HSV-1 and for the following 7 days after inoculation. Neuronal cultures were infected with approximately 10 pfu HSV-1 (17+) per neuron (m.o.i. = 10). The cells were incubated with the virus for 1 h and subsequently washed to remove any virus that did not absorb during the incubation period. In the presence of acycloguanosine, HSV-1 establishes a latent infection and can be detected as an episome in the nucleus (Wilcox et al., 1997). Furthermore, we have previously observed the presence of LAT in the HSV-1 latently infected DRG cultures (Doerig et al., 1991).

Reactivation of latent HSV-1. Following the 14-day latent infection period, the cultures were reactivated with various stimuli. Reactivation stimuli for latent HSV-1 cultures included capsaicin treatment and heat shock. Capsaicin (Alexis Biochemicals) was diluted in the neuronal maintenance medium at concentrations ranging from 0-01 to 10 μM and incubated for 4 days prior to harvesting for plaque assays. For heat-shock treatments, the latently infected neurons were placed in a 42–46°C water bath for 15 min and transferred back to a 35°C incubator for 4 days prior to harvesting the cells and medium for plaque assays. For VR-1 inhibition experiments, cultures were pretreated with the capsaicin receptor antagonist capsazepine (Alexis Biochemicals) at 10, 25 and 50 μM for 1 h prior to heat shock at 46°C for 15 min. For forskolin-induced reactivation, the neurons were pretreated with either 50 μM capsazepine or 100 μM BAPTA-AM [1,2-bis(o-aminophenoxo) ethane-N,N,N’,N’-tetraacetic acid tetraacetoxymethyl ester] and subsequently reactivated with 100 μM forskolin.

Previous experiments characterizing the in vitro latency model have determined that infectious viral particles are present in the cell lysate at a minimum of 3 days following a reactivation stimulus and that maximum virus output occurs at 5 days post-reactivation stimulus, depending on the reactivation stimulus (Wilcox et al., 1990).

Because HSV-1 is cell-associated, assaying the cell extract and medium together was necessary in order to detect total infectious viral particles present following reactivation. For gentle release of infectious viral particles from the cells, the neurons were placed in a −70°C freezer overnight. The neurons were subjected to a freeze–thaw cycle and the cell medium and extract were assayed for the presence of infectious virus. Neuronal cultures were harvested at the times indicated following the treatments indicated and plaque assays were performed. A standard plaque assay with Vero cells was used to determine infectious viral particles present following the reactivation stimuli. The results presented were from three independent experiments with six separate culture wells for each treatment group (n = 6). The data were analysed by ANOVA with a Tukey–Kramer adjustment using SAS statistical software.

Plaque assay. To determine infectious viral particles present in the cell extract and medium of the reactivated neurons, a standard plaque assay was performed. Vero cells were plated on to 24-well plates and grown to confluency. The growth medium was removed and an aliquot of 200 μl of the neuronal reactivated cell extract, at various dilutions, was incubated with the Vero cells for 1 h. Following incubation, the DRG cell extract was removed and a 1% methylcellulose-containing medium was placed on the cells. Plaque formation occurred within 3 days of the initial infection. In order to visualize the plaques, the methylcellulose was removed and the cells were fixed and stained with 1% crystal violet in methanol. Plaques were visualized under a light box and counted for each reactivated neuronal well.

Cell viability. Cell viability was determined using AlamarBlue (BioSource Int.) colorimetric dye according to the manufacturer’s protocol, which is based on an oxidation–reduction reaction. The cells were incubated with capsaicin at 1–10 μM for 4 days and then treated with AlamarBlue for 3 h. Following AlamarBlue treatment, the medium was collected and read on a colorimetric plate reader at 550 and 600 nm. All treatment groups were compared with control (no treatment) and represented as a percentage of control cultures.

Immunocytochemistry. Neuronal cultures were fixed in 4% paraformaldehyde overnight at 4°C. Following fixation, the cells were permeabilized with 0.2% Triton X-100 (Sigma) and blocked with 5% non-fat milk in PBS containing 0.5% Tween 20 for 1 h. The cells were then incubated with capsaicin receptor primary polyclonal rabbit antibody (Ab-1) at a concentration of 0.1 μg mL⁻¹ (Oncogene Research) overnight at 4°C. Following primary antibody incubation, the cells were washed and incubated with a universal secondary IgG anti-rabbit antibody conjugated to horseradish peroxidase for 1 h, according to the manufacturer’s protocol. Diaminobenzidine was used as the substrate (Vector Laboratories).

RESULTS

Detection of VR-1 in neuronal cultures

In order to examine the percentage of nociceptor-positive neurons in the neuronal cultures, we utilized immunocytochemistry to detect VR-1. The staining indicated that
approximately 90% of the neuronal cells in each culture well stained positive for VR-1 (Fig. 1A). Additionally, VR-1-positive staining was present not only on the cell body of the neurons but also on the neurites and the nerve terminals (Fig. 1D). It appeared that smaller-diameter neurons in general stained positive for VR-1 and that the neurons that did not stain positive for VR-1 were generally cells with larger-diameter cell bodies. Furthermore, staining of the adult DRG compared with embryonic DRG indicated a difference in overall abundance of VR-1 in embryonic tissue compared with adult tissue (Fig. 1B, C). In the adult DRG, the small neurons stained positive for VR-1 whereas the larger neurons did not stain, consistent with previous observations that smaller-diameter neurons correspond to nociceptors or C and Aδ fibre pain neurons (Jancso et al., 1967). As observed in the neuronal cultures, most of the cells in the embryonic whole DRG stained positive for VR-1 (Fig. 1B).

**Capsaicin treatment induces reactivation of latent HSV-1**

To determine the effects of VR-1 activation during a latent HSV-1 infection, neurons were treated with capsaicin, a potent agonist of the VR-1 channel. In the *in vitro* neuronal model of HSV-1 latency, capsaicin treatment caused

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**Fig. 1.** VR-1 staining in DRG cultures. Immunocytochemistry using anti-VR-1 polyclonal antibody to demonstrate the abundance of VR-1-positive neurons in the DRG culture (40 x) (A). In addition to the cell bodies staining positive for VR-1, the neurites and the nerve terminals were also VR-1-positive (40 x) (D). Adult DRG staining shows that smaller-diameter neurons stain positive for VR-1 whereas larger-diameter neurons do not (20 x) (C). Embryonic whole DRG were sectioned and stained for VR-1. Consistent with the cultures, most of the neurons in the embryonic (E-16) whole DRG stained positive for VR-1 (20 x) (B).
reactivation in a dose-dependent manner at concentrations of 0.01 μM with a mean virus titre of 31 183 p.f.u. ml⁻¹ to 0.1 μM with a mean virus titre of 72 588 p.f.u. ml⁻¹ (Fig. 2). However, at high concentrations of capsaicin (1 and 10 μM) there was a significant decrease in reactivation following treatment, with virus titres of 22 250 p.f.u. ml⁻¹ and 1001 p.f.u. ml⁻¹, respectively. Capsaicin treatment thus produced a bell-shaped dose-response curve for HSV-1 reactivation. To determine whether this decrease in reactivation at the higher concentrations of capsaicin was due to cytotoxicity, a viability assay was performed. The AlamarBlue viability assay indicated that no significant cell death occurred following capsaicin treatment at concentrations of 0.1, 1 and 10 μM over the 4-day treatment period (Fig. 3). There was a slight decrease in cell viability at the higher concentrations, with a viability of 87.7% for 1 μM capsaicin and 90.9% at 10 μM capsaicin when compared with control untreated cultures. However, this slight decrease in viability is unlikely to account for the decrease in virus titres observed at these concentrations.

**Heat-shock-induced reactivation of latent HSV-1 is temperature-dependent**

To examine the effects of heat-shock stimulus on neuronal cultures harbouring latent HSV-1, cultures were subjected to a heat stimulus ranging from 42 to 46 °C. Optimal VR-1 activation normally occurs at temperatures exceeding 45 °C. Following heat stimulation at 46 °C (532 500 p.f.u. ml⁻¹), we observed a 5000-fold increase in virus titre compared with 42 °C (109 p.f.u. ml⁻¹) (Fig. 4). Reactivation titres of this magnitude have never previously been observed in our laboratory. However, reactivation was not induced at temperatures below 42 °C, including the control cultures maintained at the normal culturing temperature of 35 °C. Temperatures of 44 and 45 °C produced an approximately 400-fold increase in virus titre compared with 42 °C, with virus titres of 43 666 p.f.u. ml⁻¹ and 42 500 p.f.u. ml⁻¹, respectively (Fig. 4). Thus, reactivation of latent HSV-1 was temperature-dependent with the greatest virus titre of 532 500 p.f.u. ml⁻¹ produced at 46 °C. Because nociceptors are activated at temperatures of 46 °C and greater, these data suggest the involvement of VR-1 in the induction of reactivation of latent HSV-1.
Capsazepine blocks heat-shock-induced reactivation of latent HSV-1

To determine specifically the role of VR-1 in heat-shock-induced HSV-1 reactivation, the VR-1 antagonist capsazepine was used to block heat-shock-induced reactivation. Neuronal cultures harbouring latent HSV-1 were treated for 1 h with capsazepine prior to heat shock at 46˚C. Following heat shock, the neurons were assayed for infectious virus. The data indicated that treatment with capsazepine significantly reduced reactivation of latent virus at concentrations of 25 μM ($P = 0.025$) and 50 μM ($P = 0.045$) compared with control cultures (Fig. 5). These data further suggested that reactivation following heat treatment was mediated through the activation of the VR-1 receptor.

BAPTA reduces capsaicin-induced HSV-1 reactivation

To determine further the mechanism of capsaicin-induced HSV-1 reactivation, we tested the role of Ca\textsuperscript{2+} as a mediator of this reactivation stimulus. The neurons were pretreated with the calcium chelator BAPTA-AM, prior to capsaicin treatment. BAPTA-AM is a membrane-permeable form of BAPTA that is hydrolysed by cytosolic esterases and trapped intracellularly as an active Ca\textsuperscript{2+} chelator. Seventy-nine percent of the capsaicin-treated cultures reactivated following treatment, whereas only 21% of the capsaicin plus BAPTA-treated cultures reactivated (Table 1). These data imply that capsaicin caused increases in intracellular Ca\textsuperscript{2+} essential for HSV-1 reactivation that could be attenuated by treatment with BAPTA-AM.

Table 1. Calcium mediates capsaicin-induced HSV-1 reactivation

Capsaicin-induced HSV-1 reactivation could be reduced using BAPTA-AM at a concentration of 100 μM. These data are presented as a percentage of HSV-1-reactivated cultures representing three independent experiments.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>% Reactivated (no. of positive cultures/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsaicin (0-1 μM)</td>
<td>79% (11/14)</td>
</tr>
<tr>
<td>Capsaicin (0-1 μM) + BAPTA</td>
<td>21% (3/14)</td>
</tr>
<tr>
<td>Latent, no treatment</td>
<td>9% (1/11)</td>
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HSV-1 reactivation induced by forskolin is significantly attenuated following BAPTA and capsazepine treatment

We hypothesized that forskolin may regulate VR-1 by phosphorylation, increasing the probability of activating and opening the channel, increasing calcium influx. Hence, forskolin may increase calcium levels via a putative phosphorylation event and we would expect that chelating calcium or inhibiting VR-1 would change the outcome of HSV-1 reactivation via forskolin stimulation. Forskolin stimulates adenylate cyclase activity resulting in elevated intracellular levels of protein kinase A (PKA) in addition to increasing intracellular cAMP. Elevated PKA levels have previously been associated with VR-1 activation (De Petrocellis et al., 2001). To test this hypothesis we examined the effects of the calcium chelator BAPTA, following forskolin-induced HSV-1 reactivation. Our studies determined that administration of BAPTA together with forskolin treatment reduced virus titres from 168 500 to 74 000 p.f.u. ml\textsuperscript{-1} in the in vitro latency model (inhibition of 45%) (Fig. 6). These results indicated that forskolin-induced reactivation was dependent on increases in intracellular calcium levels and therefore depleting the

Fig. 5. Inhibition of VR-1 reduces heat-shock-induced HSV-1 reactivation. Latently infected DRG cultures were pretreated with the VR-1-specific inhibitor capsazepine prior to heat-shock-induced reactivation of HSV-1. The data indicate that heat-induced reactivation was inhibited following capsazepine treatment at concentrations of 25 and 50 μM. Statistical significance using a one-way ANOVA between no treatment control and 25 μM (*, $P = 0.025$) and 50 μM (**, $P = 0.045$) was observed.

Fig. 6. Inhibition of VR-1 and Ca\textsuperscript{2+} both decrease forskolin-induced HSV-1 reactivation. DRG latently infected cultures treated with forskolin at 100 μM induced HSV-1 reactivation that was attenuated with both BAPTA-AM (100 μM) and capsazepine (CPZ; 50 μM) treatment.
available intracellular calcium with BAPTA significantly attenuated forskolin-induced HSV-1 reactivation. These data also suggest that forskolin may regulate VR-1 activity via phosphorylation of the channel, ultimately resulting in increased calcium influx in the neuron necessary for HSV-1 reactivation.

To test further the hypothesis that forskolin increases intracellular calcium levels via activation of VR-1, sufficient to cause HSV-1 reactivation, we pretreated the neurons with the VR-1 inhibitor capsazepine prior to forskolin treatment. This experiment demonstrated that forskolin-induced reactivation was almost completely blocked following treatment with capsazepine, resulting in a 97% inhibition, with virus titre levels of 5350 p.f.u. ml\(^{-1}\) compared with 168500 p.f.u. ml\(^{-1}\) with forskolin alone (Fig. 6).

**DISCUSSION**

HSV-1 reactivation has been studied in many *in vivo* and *in vitro* models but the mechanism underlying the critical pathways involved in the switch between latent and reactivated virus is not yet understood. Studies presented here indicate that the VR-1 signalling pathways may play a key role in this switch from a latent infection to reactivation. VR-1 is an ion channel that preferentially fluxes calcium in response to capsaicin, heat exceeding 45°C and a pH of 4–6, as well as other noxious stimuli. This receptor is primarily found on nociceptor neurons, which are part of the peripheral sensory nervous system. Nociceptor neurons are important for the sensation of different pain stimuli including high intensity mechanical stimulus, heat exceeding 45°C, pH changes and noxious compounds, such as capsaicin. Additionally, nociceptor neurons exist in the dermal epithelium as free nerve endings, making them more susceptible to virus uptake compared with other sensory neurons, which have a protective laminate surrounding the nerve terminal. The neuronal cultures derived from the rat DRG are a mixed neuronal population that contain nociceptor neurons as well as other sensory neurons, although our results demonstrate that the vast majority of neurons in culture are VR-1-positive.

In our studies, heat, a potent agonist of the VR-1 channel, induced HSV-1 reactivation *in vitro*. In humans, stimuli associated with heat such as fever, sunburn and burns also correlate with HSV-1 reactivation (Boak *et al.*, 1934; Bourdarias *et al.*, 1996). Based on these observations, animal models were developed utilizing heat shock as a method of inducing reactivation of latent HSV-1 (Epstein, 1941; Moriya *et al.*, 1994; Sawtell & Thompson, 1992). The mechanism of heat-induced reactivation of HSV-1 has not been elucidated but a possible model may involve over-stimulation of the heat-sensitive neurons or nociceptors, inducing reactivation of latent HSV-1. Interestingly, there are no studies to date examining the signal transduction pathways of HSV-1 reactivation following stimulation of the heat-sensitive receptor, VR-1. Our data suggest that the mechanism of heat-induced reactivation involves the activation of VR-1, since the most robust reactivation occurred at the optimal temperature for VR-1 activation, 46°C. Furthermore, this heat-induced virus reactivation could be significantly attenuated using an antagonist to VR-1, capsazepine. Although capsazepine has been utilized as a specific VR-1 antagonist, it also blocks voltage-activated calcium channels in DRG cultures (Docherty *et al.*, 1997).

Another potent agonist of VR-1 that also induces HSV-1 reactivation in the *in vitro* model is capsaicin, the active component in hot chilli peppers. Capsaicin has been previously tested as a treatment to alleviate the recurrence of herpetic lesions in an HSV-2 guinea pig latency model (Bourne *et al.*, 1999; Stanberry, 1989). Although these data indicate a slight decrease in herpetic lesions following primary infection and a decrease in immediate recurrence following capsaicin treatment, the animals became desensitized to the capsaicin treatment (Stanberry, 1989). This implies that the nerve termini may have been damaged or destroyed following capsaicin treatment. In our latency model, VR-1 activation with capsaicin induced HSV-1 reactivation from latently infected neuronal cultures. Interestingly, capsaicin induced a bell-shaped dose-response curve with decreases in virus titre at higher concentrations of capsaicin that were not due to neuronal cell death, based on the viability assay. We attributed this decrease in reactivation to desensitization of VR-1 at higher doses of capsaicin, as previously observed *in vivo* (Bourne *et al.*, 1999; Stanberry, 1989).

The desensitization of VR-1 at higher concentrations of capsaicin suggests a protective mechanism of the neuron to shut off damaging Ca\(^{2+}\) influxes. Other investigators have noted desensitization of VR-1 following repeated stimulation with capsaicin in electrophysiology experiments (Docherty *et al.*, 1996; Koplas *et al.*, 1997; Piper *et al.*, 1999). In these experiments, repeated exposure to capsaicin decreased and eventually abolished calcium currents. Furthermore, other studies have noted a decrease in mRNA levels of VR-1 following capsaicin treatment, suggesting down-regulation of the receptor (Mezey *et al.*, 2000). The mechanism underlying this desensitization is unknown, although it has been suggested that capsaicin desensitization may be similar to calcium-dependent inactivation of the N-methyl D-aspartate receptor (Docherty *et al.*, 1996). This assumes that a particular threshold of intracellular Ca\(^{2+}\) levels negatively affects channel activity and gating (Koplas *et al.*, 1997). Because capsaicin treatment results in rapid VR-1 desensitization in electrophysiology experiments and long-term desensitization in animal models, there may be two components of this desensitization. One component may be attributed to multiple ligand binding domains within particular regions on the receptor that change the kinetics and gating properties of the channel. The second component may involve receptor turnover. These two components may regulate the internal calcium levels to ensure survival of the neuron.
The neuron relies on calcium channels and non-selective ion channels such as VR-1 to regulate intracellular calcium. Hence, VR-1 is found not only at the nerve terminal, but throughout the axon and soma (Guo et al., 1999). Additionally, the neuron expresses calcium-binding proteins capable of chelating excess toxic levels of calcium, preventing continuous stimulation and allowing cellular recovery (Berridge, 1998). Prolonged Ca\(^{2+}\) increases due to a high-intensity stimulus, such as a pain stimulus, causes hyperexcitation of the neuron. This high-intensity stimulus causes a prolonged increase in intracellular Ca\(^{2+}\), creating a wave of calcium that eventually reaches the soma or cell body of the neuron (Usachev & Thayer, 1999). In the in vitro model, this hyperexcitation may occur rapidly due to the fact that we are stimulating not only the nerve terminal but also the cell body. When hyperexcitation of a neuron occurs, these unusually elevated intracellular calcium levels in the soma transition the transcriptional machinery from a normal basal level to a highly active level (Buonanno & Fields, 1999; West et al., 2001). Perhaps in response to this increase in transcriptional activity in the neuron due to prolonged Ca\(^{2+}\) stimulation, HSV-1 is able to induce the transcription of immediate-early genes and begin a lytic infection. This may occur either via a direct role through Ca\(^{2+}\) -dependent transcription factors or through an indirect activation of host-cell gene transcriptional machinery that serendipitously initiates HSV-1 transcription.

Interestingly, many of the reactivation stimuli tested in the in vitro model modulate VR-1 activation. For example, forskolin-induced HSV-1 reactivation is believed to involve the activation of PKA, which activates the VR-1 receptor (Lapshrike & Nicol, 1998). In our experiments, the calcium chelator BAPTA reduced virus titres twofold compared with forskolin alone. These data imply that forskolin increases intracellular calcium, possibly by modulating VR-1 channel activity via phosphorylation ultimately resulting in HSV-1 reactivation. In addition, pretreatment with the VR-1 antagonist capsazepine, prior to forskolin treatment, attenuated and in some cases completely abolished HSV-1 reactivation. These data strongly suggest that forskolin modulates VR-1 gating properties and channel activity. Therefore, forskolin not only increases PKA levels that may be essential for the phosphorylation of important regulatory proteins involved with the initiation of transcription of HSV-1 immediate-early genes, but also regulates VR-1 activity critical for HSV-1 reactivation. These data further support the hypothesis that VR-1 receptor activation is the mechanism of forskolin-induced HSV-1 reactivation of latently infected neuronal cultures.

HSV-1 reactivation is a complex phenomenon that may involve multiple second messenger pathways. However, there may be a common target that funnels all the different stimuli for reactivation towards a common event of VR-1 activation. Previous studies have demonstrated that upregulation of protein kinase C (PKC) and PKA caused reactivation of latent HSV-1 (Smith et al., 1992; Wilcox et al., 1990). Activation of both of these kinases can directly affect VR-1 activity. Phorbol 12-myristate 13-acetate activates the second messenger PKC that directly stimulates sensory-specific sodium channels causing excitation of the neuron, induces HSV-1 reactivation and also activates VR-1 (Cesare et al., 1999; Khasar et al., 1999; Premkumar & Ahern, 2000; Smith et al., 1992; Vellani et al., 2001). Additionally, excitation of a nociceptor via direct activation of VR-1 with heat or capsaicin causes depolarization and firing of the neuron (Caterina et al., 1997). Here we have shown that treatment of latently infected neurons with capsaicin or heat shock also induces reactivation of HSV-1 latently infected neurons in culture. A common feature of all of these reactivation stimuli is hyperexcitation of the neuron resulting in elevated intracellular calcium caused by VR-1 activation.

In conclusion, overstimulation of a nociceptor neuron sufficient to activate VR-1 is critical for the induction of HSV-1 reactivation. Our data support the hypothesis that a pain stimulus sufficient to activate VR-1 may create the switch from a latent HSV-1 infection to reactivation.

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