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DISSERTATION

SIGNALING MECHANISMS ESSENTIAL FOR REACTIVATION OF LATENT HERPES SIMPLEX VIRUS TYPE 1 IN NEURONAL CULTURES

Submitted by
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In partial fulfillment of the requirements
For the Degree of Doctor of Philosophy
Colorado State University
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ELIZABETH A. HUNSPERGER ENTITLED *SIGNALING MECHANISMS ESSENTIAL FOR REACTIVATION OF LATENT HERPES SIMPLEX VIRUS TYPE 1 IN NEURONAL CULTURES* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

SIGNALING MECHANISMS ESSENTIAL FOR REACTIVATION OF LATENT HERPES SIMPLEX VIRUS TYPE 1 IN NEURONAL CULTURES

Herpes simplex virus type 1 belongs to the herpesviridae sub-family alphaherpesvirinae. This family of viruses is recognized by their ability to establish a life-long latent infection in humans. Although generally not life threatening, HSV-1 has adapted to its human host with perfect precision. Latency and reactivation remain a mystery to scientists to date. The only cells that establish a latent infection are sensory neurons. This cell type specificity and the mechanisms of reactivation remain unclear. The experiments and studies presented here attempt to elucidate a potential unifying hypothesis of herpes reactivation that involves calcium modulation in the nociceptor neuron.

Calcium is a highly regulated ion in neurons with pleiotropic effects. The diverse roles of calcium in the neuron allow it to change the membrane potential towards depolarization and also signal apoptosis. Many of the known stimuli both in humans and in our culture system cause an increase in intracellular calcium levels and ultimately lead to herpes reactivation. These secondary pathways include activation of protein kinase C (PKC), protein kinase A (PKA), vanilloid receptor-1 (VR-1) activation, nerve growth factor (NGF) deprivation and heat shock.

Studies presented here focused on the pain receptor, VR-1. VR-1 is a calcium ion channel found primarily on nociceptor neurons. Its activation results in herpes

reactivation that is reversible with the specific inhibitor to VR-1, capsazepine. We tested two different agonists, heat stimulus exceeding 45°C and capsaicin. Both agonists caused herpes reactivation with different kinetics. Capsaicin treatment resulted in a bell-shaped dose response curve that could be attributed to desensitization of the channel. Whereas heat stimulation yielded significant increase in reactivation for temperatures that were permissive for VR-1 activation.

Additionally, NGF withdrawal experiments indicated that the pro-apoptotic protease enzyme, caspase-3 plays a pivotal role in herpes reactivation. Caspase-3 exists as a pro-enzyme in the cytoplasm of the cell and upon activation to its catalytic form, it translocates to the nucleus where its major role in DNA fragmentation takes place. Our studies showed that inhibition of caspase-3 following NGF deprivation attenuated but did not abolish HSV-1 reactivation. In order to address the direct role of caspase-3 in HSV-1 reactivation, we treated the neurons with C2-ceramide and over-expressed caspase-3 with a recombinant adenovirus. Both of these treatments induced HSV-1 reactivation in our model suggesting a direct role of caspase-3.

In conclusion, calcium homeostasis is essential for neuronal survival, signaling and plasticity. Disruption of this homeostasis through various stimuli that excite the nociceptor neuron may lead to reactivation. This disruption modulates neuronal excitability rendering the neuron more susceptible to herpes reactivation in response to a milder stress stimulus.

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Chapter 1

Introduction

1. Historical Perspective of an old Enemy Within

Adaptation and fitness to an environment is one of the key elements of survival and promotion of the species. Viruses are able to adapt and fit a host better than any organism. An example of this adaptation and long-term survival is herpes simplex virus type 1 (HSV-1). HSV-1 was historically first described and named "herpes" by the Greeks 25 centuries ago. Herpes signifies to creep or crawl. This nomenclature described the insidious nature herpes spreads from different regions of the body and the transmission from individual to individual. The first documented description of herpes fibrilis or fever blisters was by Herodotus approximately A.D. 100, when he made the astute correlation between the occurrence of fevers and the onset of mouth ulcers and lip lesions. Later, this Roman scholar named the disease herpes fibrilis. Further description was provided in the writings of Hippocrates, the father of medicine. In these earlier times, herpes lesions were believed to be an attempt of the body to rid itself of evil humors. Shakespeare himself made references to herpes in several of his well-known plays such

as in Romeo and Juliet. Furthermore, Astruc, the physician to the King of France, documented genital herpes as early as 1736 (1).

In 1893 an important discovery by Vidal recognized that human transmission and spread of herpes occurred through physical contact (88). In the 1920's Doerr and Vochting demonstrated that herpes could spread from the cornea to the brain (18). Following these studies, in 1923, Goodpasture and Teague suggested that viral spread occurred by intra-axonal pathways (28). Forty-five years later, in 1968, there was a distinction made between HSV-1 and type 2 based on both biological behavior and serological differences (61). Additionally, during this time it was suspected that recurrent herpes infections were due to reactivation of dormant or latent herpes but not yet proven. Three years later, in 1971, Stevens and Cook proved that herpes latency occurred in dorsal root ganglion cell bodies of the peripheral nervous system (78).

2. Herpes Replication During a Productive Infection

Herpes simplex virus type-1 (HSV-1) belongs to the *Herpesviridae* family of viruses. This family of viruses is divided into three different major subfamilies; alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. HSV-1 is classified as an alphaherpesvirinae and it is a linear, double stranded, enveloped, DNA virus containing an icosahedral shaped nucleocapsid (64). The viral genome consist of 152kbp with approximately 68% G + C content (42). The genome is divided into two segments that are called the Unique Long (UL) and the Unique Short (US) regions. Flanking both of these segments are inverted repeats referred to as Repeat Long (RL) and Repeat Short RS) respectively, as diagramed in Fig.1.1, and genes located in inverted repeat regions

are present in two copies (22). There are 80 or more genes that this genome encodes and each gene has its own promoter (54).

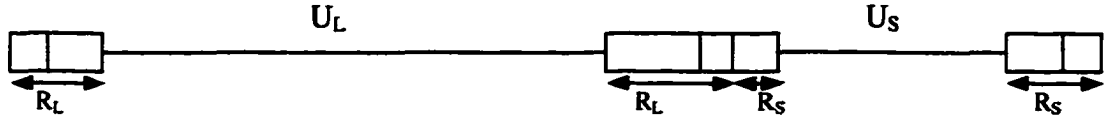


Fig. 1.1 Schematic representation of the HSV Genome

The primary infection begins with the attachment of the virus to the host cell heparin sulfate proteoglycans on extracellular matrix, with viral membrane protein interactions usually involving gB and gC (12). Another viral attachment protein involved in viral penetration is gD, which interacts with a host secondary cellular receptor (11). The cellular receptors believed to be most essential in neurons appears to be HveC (data unpublished). Contact between the primary and secondary receptor initiates fusion of the virus with the host cell and absorption. Absorption and penetration of the virus requires gB, gD and gH. Upon absorption, the capsid and tegument proteins are released into the cytoplasm and the capsid is transported to the nucleus (82). Capsid transport occurs via microtubule interaction with the viral nucleocapsid (47). Through an unknown mechanism, the viral DNA is injected into the nucleus through the nuclear pore. The viral DNA becomes circularized upon entry into the nucleus and a rolling circle-like replication mechanisms results in concatemers that are cleaved into monomers and packaged into pre-formed capsids (6, 36).

Our understanding of the overall pattern of viral gene expression has remained unchanged for 20 years, with first the expression of immediate early (IE) genes (alpha genes) followed by the expression of early genes (beta) and late genes (gamma). The IE

genes are regulated by various cis-acting elements that include a TATA and CAAT box. In addition, there are transcriptional SP1 binding regions within these IE promoter elements. The IE genes were originally defined as genes that were expressed in the absence of *de novo* protein synthesis. The alpha gene products included Infected Cell Protein 4 (ICP4), ICP27, ICP0, ICP47, and ICP22. The expression of these gene products is regulated and controlled by the transactivator VP16. Approximately 500-1000 copies of VP16 are carried into the cell as part of the tegument protein upon viral entry (31, 74). VP16 does not directly bind DNA and it requires the presence of host cell factor (HCF) and the host transcriptional activator Oct-1 to form a complex on the regulatory sequence TAATGARAT to be active. All of these factors act in concert to recruit RNA polymerase II transcriptional machinery and begin viral translation of the immediate early proteins. Maximal expression of alpha genes occurs between two to four hours following viral entry.

Beta gene expression follows the alpha genes and encodes essential proteins necessary for nucleotide metabolism including thymidine kinase (tk, UL23). Tk is the most commonly studied viral gene (55, 56). The tk gene promoter element requires common eukaryotic cis-acting elements for its activation and expression. The mechanism of its regulation remains a mystery since this gene is only turned on during early gene expression and is turned off following DNA synthesis. The product of the tk gene cultivated the development of the first anti-viral pro-drug, acyclovir first discovered in 1974 by a drug-screening program at Burroughs Wellcome. Following its discovery, acyclovir was not commercially available until 1982 (43). Tk phosphorylates acyclovir to a nucleotide analog that competes with guanosine. Viral DNA polymerase attempts to

incorporate acycloguanosine into the newly synthesized viral DNA strand causing the polymerase to stall and terminate synthesis. In the absence of viral tk, acyclovir cannot be metabolized into a nucleotide analog therefore it does not interfere with normal host cell division. Currently, the only effective anti-viral drugs available for the treatment of HSV-1 target viral thymidine kinase.

The gamma genes require synthesis of viral DNA to be fully activated and encode the structural proteins necessary for assembly and packaging of infectious virus particles (33). Many of the products of the alpha genes are essential for activation of these late gene products including, ICP4 (84). Interestingly, late genes do not require cis-acting regulatory elements (32). Additionally, both late genes and early genes are rarely spliced following transcription. Often their regulatory promoters are downstream from the TATA box.

Replicated viral DNA is packaged into formed capsid proteins. The viral DNA is packaged with tegument proteins, buds from the nucleus and is enveloped in the endoplasmic reticulum (ER). The movement of the virus from the ER to the plasma membrane is unclear. There are two recognized hypotheses regarding this event. The de-enveloped hypothesis states that the capsid with viral DNA fuses with the ER membrane picking up a membrane to later de-envelope into the cytoplasm and pick up an envelope upon fusion with the Golgi, that contains viral membrane proteins. Then the mature virion is transported to the plasma membrane by a vesicle and exocytosed. Conversely, in the luminal model, the enveloped virus in the ER is placed in a transport vesicle targeted to the Golgi. The Golgi is then responsible for the modification and processing of the viral envelope proteins. The difference lies in the origin of the viral membrane of the

mature virion. In the luminal model this membrane originates from the inner nuclear membrane but in the de-enveloped model the membrane originates from the Golgi (reviewed in (24)).

3. HSV-1 Latency in Sensory Neurons

Latency is defined as an infection where the viral genome is maintained in a non-replicating state in the host cell. Although HSV-1 is capable of infecting almost any cell in culture, it remains dormant or latent only in the peripheral nervous system for life-long infection (78). The mechanism that renders the virus latent in the peripheral nervous system (PNS) is still not well understood. HSV-1 gains access to nerve termini during the initial productive infection of the dermal epithelial cells. Following viral uptake in the sensory nerve termini, HSV-1 is retrogradedly transported to the cell bodies of the

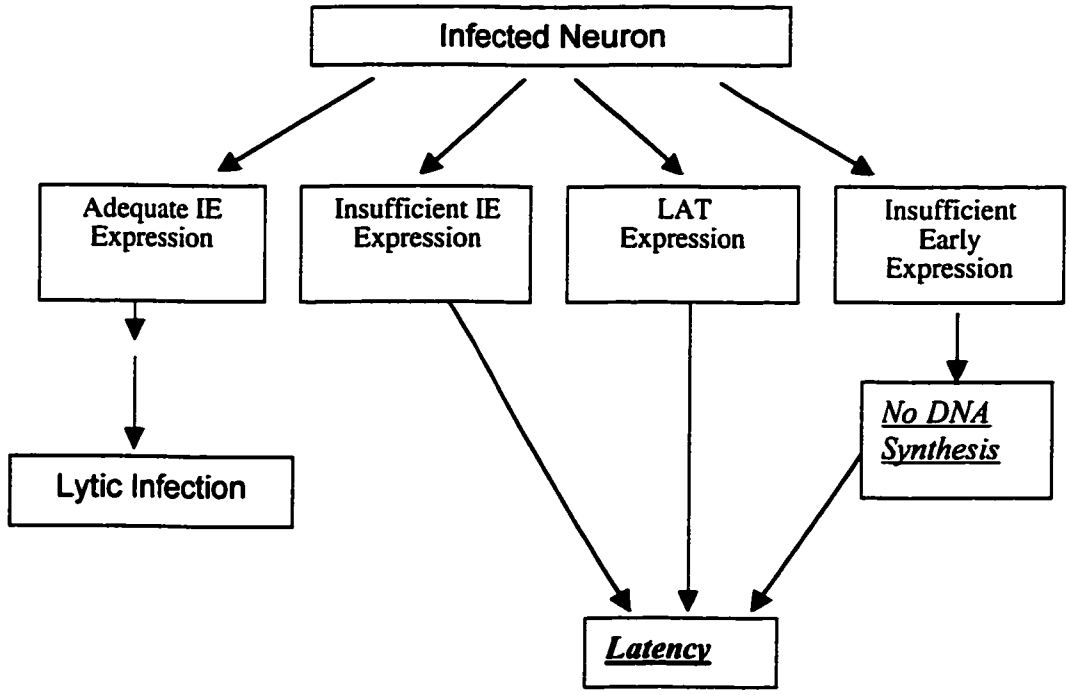


Fig. 1.2 Hypothesis of HSV latency in neurons

sensory neurons (4). The virus is transported to the nucleus of the sensory neuron, whereupon it can establish latency (77).

There are three main hypotheses for the mechanism of the establishment of latency in neurons. First, there is a very high abundance and expression of the Latency Associated Transcript (LAT) specifically in neurons. Second, it is postulated that latency occurs because of insufficient alpha gene expression. Third, there is some evidence supporting the idea that latency is established due to insufficient beta gene expression (Fig.1.2).

During a latent infection, the viral genome is maintained in a non-replicative state where the only gene expression detected is LAT. LAT is an RNA transcript first discovered by Stevens et al., whose function in maintaining latency or establishing latency is still not clear (79). LAT is only expressed in latent neurons and has become the hallmark for the identification of a latent neuron. Although *in vivo*, not all neurons that harbor HSV-1 DNA express LAT (68). It is not clear whether LAT is responsible for latency in neurons or whether LAT maintains the latent infection. The translation of the RNA transcript LAT into a protein product is still debatable. Wilcox et al., 1994 is the first known publication regarding LAT protein detection in sensory neurons. Six years later, other investigators attempted to over-express the putative LAT protein to determine its role in the herpes life cycle. No other publications to date have reported the presence of the LAT protein in latent neurons. Albeit, LAT's function in maintaining latency remains unclear, its expression has been detected in over 90% of latent DRG cultures (72). In latent cultures, HSV-1 reactivation induced by NGF deprivation causes rapid

LAT translocation from the nucleus to the cytoplasm (16). These data suggest that LAT is important in maintaining latency and viral reactivation.

The immediate early or alpha gene products are of great importance to the hypothesis of latency in neurons. It is believed that HSV-1 establishes latency in neurons because of insufficient expression of the alpha genes following the initial infection. HSV-1 does not replicate very efficiently in neurons and this inefficiency may involve altered expression of the alpha genes. This decrease of necessary viral transcription factors and other essential proteins in the neuron disrupts efficient expression of the immediate early genes. This disruption may transition the virus from a productive infection to a latent infection (85). Furthermore, because the capsid and tegument proteins require active transport to the nucleus from the distal periphery, it has been speculated that VP16 and other immediate early genes do not arrive at the nucleus and the viral DNA cannot initiate gene expression. Moreover, in vitro studies by Kemp et al., and Lillycrop et al., indicated that neurons actually contain inhibitory octamer proteins that may prevent the scaffolding of Oct-1/Host Cell Factor/VP16 from occurring therefore driving the virus towards a latent infection (40, 52).

Additionally, there is recent evidence that ICP0 may play an important role in the establishment of latency. ICP0 is expressed at a very low level or even repressed in neurons (Wilcox et al., unpublished data). This may be due to differences in the presence of nuclear structures such as promyelocytic leukaemia (PML) or nuclear domain-10 (ND10) in neurons versus actively dividing cells. Data from our laboratory have shown that ND10 and PML expression in neurons is absent. These structures are thought to encompass important transcriptional active zones essential for herpes replication (53).

ICP0 is known to associate to these regions during productive infection. Interestingly, when using a recombinant adenovirus over-expressing ICP0-GFP in our neuronal model, we observed very low expression using fluorescent microscopy nevertheless we were able to locate the protein by western gel analysis (personal communication, Sarah Richart). ICP0 is either destroyed or translocated to the cytoplasm for rapid degradation. This preliminary data implies that ICP0 is degraded rapidly in neurons because of the lack of nuclear domains (ND10) and that this degradation contributes to neuronal specific latency.

The third theory of latency involves the insufficient expression of early (B) genes. This theory actually relies on the first theory of insufficient alpha genes. Because of the lack of sufficient alpha gene products, the beta genes are unable to initiate viral DNA synthesis. Therefore, viral DNA synthesis is aborted and the DNA remains dormant or latent in the nucleus of the neuron. Some investigators have speculated that this is the reason behind multiple copies of HSV-1, up to 10,000 copies found in the neuron (68). Perhaps, when viral DNA synthesis is initiated and early gene products necessary for late gene expression are not present, the virus goes through multiple rounds of attempted replication that are aborted. This may explain the multiple copies of viral DNA that remain in the nucleus as episomes.

In summary, establishment of a latent infection in neurons may be due to LAT, insufficient alpha gene products or beta gene product expression and degradation of ICP0. HSV-1 evades the immune system by establishing a latent infection in sensory neurons. In humans, reactivation of latent HSV-1 can be induced by fever, stress, prostaglandin release, skin abrasion and UV irradiation. Upon reactivation of latent HSV-

1, the infectious virion is anterogradedly transported back to the dermal epithelium to begin the cycle of primary infection and possibly to re-infect other sensory neurons in the vicinity. Reactivation of latent HSV-1 does not clear the virus from the nervous system but may provide an avenue for HSV-1 to infect other sensory neurons in the vicinity. HSV-1 may be selective in the type of sensory neuron it can infect or latently infect based on its location and availability in the epithelium.

3.1 Models for HSV-1 Latency and Reactivation

Reactivation of herpes is the transition or switch from a quiescent infection to a replicative state that occurs due to many seemingly unrelated stimuli. There are only a handful of models to study HSV-1 latency and reactivation. An ideal model for latency and reactivation should recapitulate the majority of the events noted in the human condition. These events include efficient establishment of latency in the PNS that is detectable by LAT or viral DNA and efficient reactivation induced by fever, stress, UV irradiation or mechanical damage to the epithelium. There are three animal models that are accepted among herpes virologist and essentially three *in vitro* cell culture models. The animal models include a mouse model with two routes of infection including the footpad and the eye, a rabbit model and a guinea pig model (25, 62, 75, 78). The limitations of the animals models include: inefficient reactivation in the mouse model, unreliable reactivation in the guinea pig model and HSV-1 strain specific latency in the rabbit model with spontaneous reactivation.

In addition to the animal models, there are three known cell culture assays used to study HSV-1 latency and reactivation. These models include DRG cultures, PC12 cell

culture and mouse trigeminal ganglion explant (8, 58, 87). Obviously, the cell culture models also have their limitations. For example, the PC12 model has been criticized for two main reasons. First, these cells are similar to sympathetic neurons and not sensory neurons. Second, the cells do not truly become latent because infectious viral particles can be assayed during the first ten days following primary infection before becoming quiescent. We used a primary sensory neuron culture system developed by Wilcox and Johnson Jr. in 1987 (87). Most studies to date examining HSV-1 signal transduction mechanism do not use primary sensory neurons but instead rely on cell lines. Because neurons are the only cells that herpes establishes latency, our neuronal culture model closely approximates the human condition of herpes latency and reactivation. This model is an invaluable resource to study specifically the effects of neuronal signaling pathways involved in HSV-1 reactivation.

4. HSV-1 Neurotropism and the Role of Pain Neurons

In humans, HSV-1 often causes pain and occasionally hyperalgesia following viral eruptions on the epithelial surface. Upon replication, HSV-1 causes tissue damage and inflammation rendering a painful stimulus to the infected individual. Interestingly, some patients infected with HSV-1 experience pain sensation prior to herpetic lesion formation and long after the lesion disappears (51).

Nociception or pain perception is carried along two subgroups of peripheral neurons called the c-fibers and Aδ fibers. The DRG contains the cell bodies of the peripheral neurons, which extend axons to the surface of the skin to detect exteroceptive perception such as pain, touch, cold, heat and pressure. The c-fiber is an unmyelinated,

polymodal nociceptor that carries signals to the spinal cord in relation to thermoreception and nociception that are relayed to the brain. The c-fiber transmits thermoception signals associated with the detection of extreme and harmful temperatures ranging from 40°C to 60°C (reviewed in (38). These nerve terminal receptive fields are very small, sensitive to localized rapid changes in temperature and can be desensitized at a saturation point. In addition to thermal sensation, nociceptors can also respond to pH changes and noxious chemicals (37).

Nociceptor neurons are identified histologically by the small cell body size compared to other neuronal cell bodies of the DRG or trigeminal ganglia. They are also identified in electrophysiology experiments by their sensitivity to capsaicin, the active component of hot chili peppers. These nerve terminals exclusively express capsaicin receptors or the vanilloid receptor-1 (VR-1) (13). Because VR-1 normally transmits thermal information to the brain, the consumption of capsaicin-containing foods is often mistaken for the sensation of heat.

Pain neurons or nociceptors exist as free nerve termini making them most vulnerable to initial viral uptake in the dermal epithelium following HSV-1 productive infection. The pain and heat perceiving neurons are the only peripheral sensory neurons that exist as free nerve endings in the epithelium. Most of the other nerve endings in the periphery are protected from viral uptake because of their membrane-like structures surrounding the nerve terminal. For example, the Pacinian corpuscle responds to vibration stimulus and contains concentrically arranged layers of connective tissue laminae around the nerve terminal, rendering a limited access to viral uptake. Furthermore, HSV-1 preferentially localizes to PNS dermatomes corresponding mostly to

neurons from the trigeminal ganglia. The trigeminal ganglia neurons innervate the face, mouth, nose and eyes. Moreover, these dermatomes contain a dense nociceptor population compared to other dermatomes in the human body (Fig.1.3).

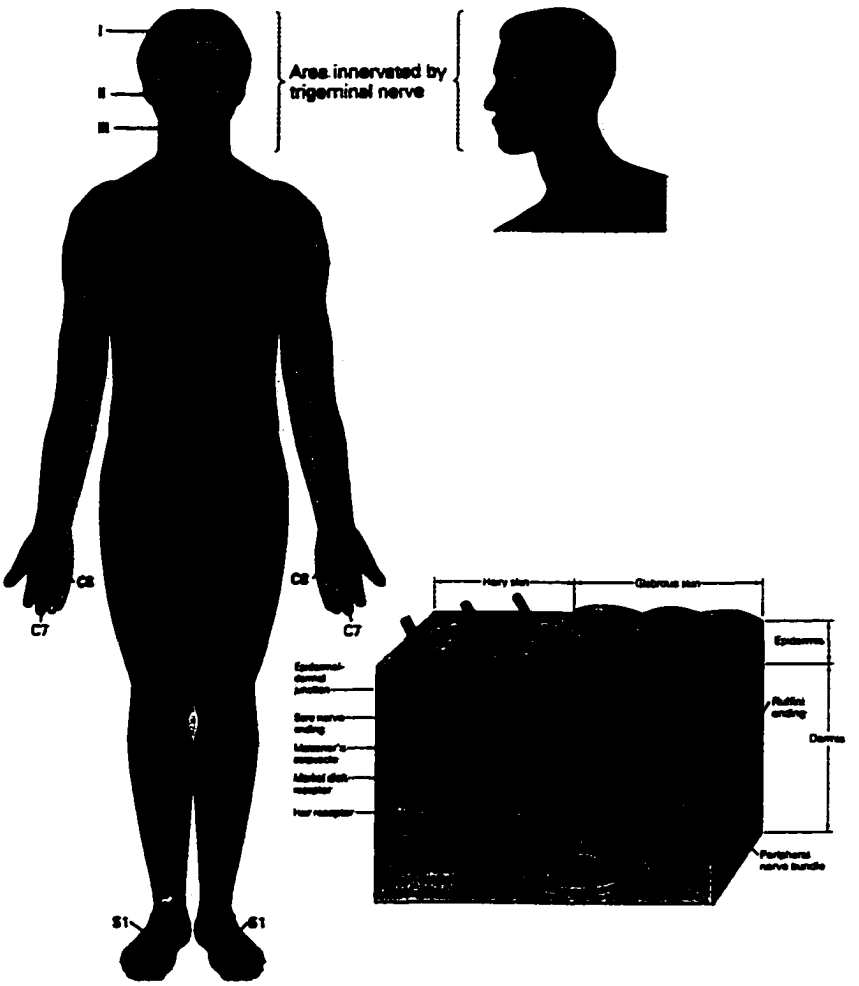


Fig. 1.3 Dermatomes corresponding to HSV infection are primarily the trigeminal dermatomes. Dermal layers demonstrating sensory neuron sub-types (38)

HSV-1 exclusively establishes latency in PNS neurons but occasionally HSV-1 gains access to the CNS causing herpes simplex encephalitis, which can be fatal. Infection of the CNS can occur via retrograde transport of HSV-1 from the peripheral nervous system to the CNS. HSV-1 has been located primarily in the temporal lobe but

based on direct connections from the PNS to the CNS this seems unlikely, yet infection of the olfactory lobe can explain temporal lobe involvement. Furthermore, viremia is not common and is not believed to be the route of CNS invasion except in neonates, where it is unclear whether this causes CNS infection. The questions that still remain unanswered are: 1.) What is the route of HSV-1 CNS invasion? 2.) Why is the virus unable to establish a latent infection in the CNS? 3.) Why does the virus begin a lytic infection in temporal lobe neurons and not the thalamus neurons, the first connection from the PNS to the CNS? Given that herpes encephalitis can be fatal, the literature in this field is sparse, hence the paucity of information regarding a heavily studied virus.

5. Herpes Reactivation in Neurons

5.1 Heat Activated Mechanisms of Herpes Reactivation

Herodotus made the astute observation that fever often accompanied herpes outbreaks and coined the term "herpes fibriles". Later in 1941 Epstein scientifically documented fever induced herpes outbreaks in humans (21). Based on this phenomenon, animal models were developed to recapitulate fever induced herpes reactivation, yet the mechanism involved is still unknown (58, 69). We hypothesize that the mechanisms of heat-induced reactivation involve over-stimulation of heat-sensitive nociceptor neurons causing toxic calcium levels. 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 or the capsaicin receptor.

The most current studies of capsaicin and herpes reported a decrease in herpetic lesions after capsaicin treatment (10, 76). These studies vaginally inoculated HSV-2 in

guinea pigs. Analysis of the data relied on visual scores to determine severity of the disease and recurrence. Although these data indicate a mild decrease in herpetic lesions following primary infection and a decrease in immediate recurrence, the animals became desensitized to the capsaicin treatment (76). This suggests nerve terminal damage following capsaicin treatment, which can lead to cell death of the virally infected sensory neuron and ultimately the destruction of latent HSV-1.

We hypothesize that HSV-1 preferentially enters the host peripheral nervous system through c-fiber sensory tracks via the VR-1 receptor and establishes latency within these cell bodies in the DRG. We propose that the VR-1 receptor plays a key role in the signal transduction mechanism of herpes reactivation because these neurons are more likely to harbor latent virus and are activated by the same stress responses that cause herpes reactivation in humans. Using this model system, the heat shock data indicated a temperature-dependent increase in viral output from HSV-1 latently infected neurons following incremental increases in temperature from 42°C to 46°C. Additionally, capsaicin stimulation initiated viral reactivation in latently infected neurons. Our data, in conjunction with previously characterized animal studies and human observations, suggest that heat stimulus is important in reactivation.

5.1a The Thermal Sensor: Vanilloid Receptor-1

The capsaicin receptor or VR-1 is a non-selective ion channel with a preference for the divalent cation, Ca^{2+} (13). An exogenous ligand of VR-1 is capsaicin, the active ingredient of a pungent plant extract of the *Capsicum* pepper. Characterization of this ion channel demonstrated that other cations flux through the channel including monovalent

cations such as Na^+ and K^+ (13). The VR-1 channel is expressed predominantly by small diameter neurons within the sensory ganglia. Activation and opening of this channel can be triggered by either heat stimuli known to elicit pain or by capsaicin binding, creating an inward current (35, 44).

VR-1 has six putative transmembrane domains and has high homology to store-operated Ca^{2+} channels called the transient receptor potential channel (TRP). Caterina et al., 1997 first cloned and characterized the VR-1 channel and determined that it is a 95 kilo Dalton protein with 838 amino acids (13). In response to stimulation, VR-1 increases the influx of extracellular Ca^{2+} due to depletion of intracellular Ca^{2+} stores. VR-1 can be selectively inhibited with a compound known as capsazepine (7). In response to ligand binding or heat stimulation exceeding 45°C, this receptor leads to the depolarization or the excitation of the neuron (45). Depending on ligand binding, this membrane depolarization may be sufficient to create an action potential to send a signal to the brain relaying the sensation of a painful heat stimulus.

5.2 Apoptotic Mechanism of Herpes Reactivation

5.2a General Anti-Apoptotic Mechanisms of Herpes

Apoptosis was defined by the Greeks to signify "leaves falling" in reference to the death of leaves on the trees during autumn. Currently, scientists use the word apoptosis to signify programmed cell death. In the field of virology, apoptosis is an important self-defense mechanism for the host cell in response to a viral infection. Many viruses inhibit the apoptotic pathway in order to sustain cell viability until full viral replication is obtained. Some of the known viral mechanisms that inhibit apoptosis normally involve

anti-apoptotic proteins encoded by the virus, for example, cytokine response modifier (Crm A) of the cowpox virus and p35 of the baculovirus (15, 67). Both of these anti-apoptotic viral proteins are specific inhibitors of the caspase class of apoptotic proteolytic enzymes essential for initiation of apoptosis.

Although many viruses have anti-apoptotic proteins or strategies, the anti-apoptotic proteins used by HSV-1 are less clear. Galvan et al., 2000 attempted to uncover the potential mechanisms involved in HSV-1 induced apoptosis in a productive infection (27). These studies indicated two separate mechanisms involving a caspase-3 dependent and independent pathway. These studies were inconclusive in elucidating the cellular proteins essential for HSV-1 induced apoptosis. Further studies by Munger et al., 2001 implicate two early gene products, US3 and US5, as important anti-apoptotic proteins expressed by HSV-1(59). These beta gene products inhibit caspase-3 and cytochrome c release from the mitochondria by some unknown mechanism. Additionally, studies by Perng et al., 2000 observed that the LAT transcript displayed anti-apoptotic activity in a rabbit latency model (63). In these studies, apoptosis was measured using PARP deactivation and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining. TUNEL staining often yields false positive results for HSV-1 infected cells because of the presence of linear HSV-1 DNA in the nucleus. Follow up studies by this group discovered through mutation analysis that particular LAT regions were anti-apoptotic and that LAT through an unknown mechanism actually inhibited Bax, a cellular pro-apoptotic protein (34).

In summary, HSV-1 may modulate apoptosis in its host cell by using the alpha gene products, ICP4 and ICP27; beta gene products, US3 and US5 and /or the LAT

transcript. All of these unrelated herpes gene products hypothetically inhibit apoptosis via inhibition of caspase-3 or up-regulation of Bcl-2.

5.2b Nerve Growth Factor Deprivation Induces Herpes Reactivation via Apoptotic Signaling

The developing nervous system relies heavily on apoptosis to shape and mold the final neuronal connections both in the peripheral nervous system and the central nervous system. Trophic and tropic factors are essential signaling molecules that determine the survival of a given neuron and its ability to enervate its target. As neurons mature their dependence on trophic factors change. The maturation of the neuron causes changes in intracellular proteins and signaling pathways that alters cell death in the absence of the growth factor (20, 29).

DRG neurons require the neurotrophic factor, nerve growth factor (NGF) for survival and maintenance (30). The binding of NGF to its receptor stimulates a series of secondary signaling pathways in the neuron. The DRG neuron expresses two diverse receptors for NGF, the high-affinity p140 and low-affinity p75 receptors. The p140 receptor is a member of the trkA (tyrosine receptor kinase) family of the receptors and signaling through the p140 receptor requires NGF dimerization (39). The binding of NGF to the p140 receptor activates two essential signal transduction pathways within the cell: the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI-3K) pathways. The MAPK pathway is initiated with the activation of ras pathway. This pathway is essential for glucose uptake, cell metabolism and cell differentiation (17). Whereas, the PI-3K pathway is important for intracellular Ca^{2+} regulation and

maintenance of Ca^{2+} homeostasis in the neuron (23). In summary, p140 receptor has two functional roles in the nervous system involving either activation of second messenger pathways essential for cell survival and/or maintenance of the neuron (Fig. 1.4).

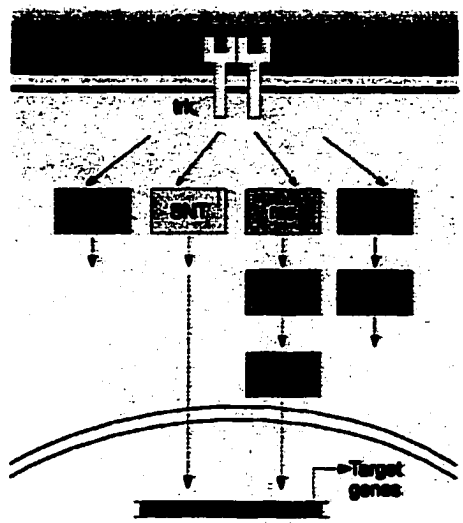


Fig. 1.4 Nerve growth factor receptor (TrkA) signal transduction pathways (38)

In contrast, the function of the NGF p75 receptor is less clear. There is evidence that this receptor has properties corresponding to a growth factor sensor. It is thought that under normal conditions NGF p75 receptor remains bound to NGF, but in the absence of NGF, p75 is activated and initiates an apoptotic signaling pathway in the cell. This theory is based upon the fact that antisense inhibition of the NGF p75 receptor prevents apoptosis in response to NGF withdrawal in DRG neurons (5). Additionally, further experiments performed by Rabidzadeh et al. (1993) demonstrated that the NGF p75 receptor induced apoptosis in the absence of NGF binding (66). Taken together these data suggest that the NGF p75 receptor is a NGF sensor that signals in the absence of NGF.

Because the NGF p75 receptor is not as critical to cell survival as the p140 receptor, it has not been the focus of the majority of the research hence there is a paucity

of information on its signal transduction mechanism. The NGF p75 receptor is in the same receptor family as the tumor necrosis factor-alpha (TNF-α) R1 and the Fas/Apo-1 receptor. This structural proximity may imply similar mechanisms of apoptotic signaling (71). Both the TNF-αR1 and Fas/Apo-1 receptors have cytoplasmic death domain regions that bind adaptor molecules, such as caspase-8 (9). If the NGF p75 receptor behaves as a NGF sensor, determining the available amount of NGF present, we postulate that the NGF p75 receptor in the absence of NGF, may directly activate the caspases. Activation of the caspases may be mediated through a putative death domain on the cytoplasmic region of the NGF p75 receptor. Hence, the lack of binding of the ligand to its receptor may initiate proteolytic activation of the caspases enzymes initiating apoptotic signaling pathways.

5.2c The Role of Caspase-3 in Apoptosis and HSV-1 Reactivation

There is an orderly initiation of cell suicide that begins with the activation of the caspase enzymes. Caspase is an acronym referring to Cysteine Aspartate Proteases that cleave between these two amino acids. These classes of enzymes exist in the cytoplasm as pro-enzymes and require cleavage to an active form. The cascade of caspase activation is often initiated as a receptor event that begins the apoptotic signaling. The caspase family of enzymes is divided into two functionally defined classes. The class I caspases are targeting molecules often associated with death domains in the cytoplasmic regions of either a TNF-R1, Fas/Apo-1 receptor or in this case the NGF p75 receptor and includes caspase 1, 2, 4, 5, 8 and 10. The class II caspases are proteolytically cleaved by the class I caspases (reviewed by (49)).

The class II caspases includes 3, 6, 7 and 9 and these enzymes are effector molecules that are downstream modulators of apoptosis. Although activation of the caspase enzymes cause many downstream events leading to apoptosis, it appears that caspase-3 specifically plays an important role in the nervous system. This key role in the nervous system became evident upon development of the caspase-3 knock-out mouse. These animals developed enlarged nervous systems and were severely compromised by the lack of normal developmental neuronal apoptosis (48).

Caspase-3 is a class II caspase enzyme cleaved by caspase-8 into its active form. Activation caspase-3 is not restricted to caspase-8 cleavage but can also occur following cytochrome-*c* release from the mitochondria. This can occur when mitochondrial function is compromised. Once caspase-3 is proteolytic cleaved, it translocates to the nucleus. Upon entering the nucleus, caspase-3 substrate targets include the cleavage and deactivation of an essential DNA repair enzyme, Poly (ADP ribose) Polymerase (PARP) and other essential proteins such as DNA protein kinase. The final downstream events following caspase-3 activation are DNA fragmentation and chromatin condensation, the hallmarks of the end stage of apoptosis.

Viral factors may be directly affected by caspase-3 activation. Immediate early (IE) regulatory factors of HSV-1 appear to control infection as repressors or activators and therefore may serve as potential targets for regulation of viral replication. Possible structural changes of the IE proteins by caspase-3 proteolysis may preclude DNA binding and inhibit IE repressor activity. Through computer analysis, we have determined that there exist several caspase-3 potential cleavage sites within HSV-1 IE gene products. These IE gene products include ICP4 and ICP27. The functional roles of these proteins

are diverse and not yet completely understood. The literature suggests that ICP27 blocks mRNA splicing mechanism but is also capable of gene regulation as a repressor (57). ICP4 binds to DNA and functions as an activator and repressor of gene expression (Koop et al., 1993). In addition, some evidence suggests that ICP4 is constitutively expressed during the latent HSV-1 infection (46). If so, ICP4 may be involved in maintaining latency. Furthermore, proteolysis of ICP4 by caspase-3 may induce HSV-1 reactivation and a possible mechanism of NGF deprivation-induced reactivation.

In productive infection both ICP4 and ICP27 are implicated as anti-apoptotic proteins. Deletion mutants that remove both copies of ICP4 from the herpes genome (d120) and ICP27 null mutants accelerate apoptosis in a productive infection. Based on these studies, ICP4 and ICP27 are thought to be important herpes anti-apoptotic protein by inhibiting caspase-3 and stabilizing Bcl-2 and p38MAPK (2, 27).

We hypothesize that ICP4 and ICP27 compete with caspase-3 host substrates. First, ICP4 has several caspase-3 cleavage sequences within its amino acid sequence. Second, activation of caspase-3 causes ICP4 breakdown that can be attenuated with caspase-3 inhibitors, suggesting that ICP4 is a caspase-3 substrate that competes with host targets. Taken together, these suggest that the virus not only uses ICP4 for its own gene regulation but also uses caspase-3 breakdown of ICP4 for its own down regulation necessary to initiate beta gene expression and repress any further alpha genes expression.

In our experiments, inhibition of caspase-3 attenuated but did not abolish HSV-1 reactivation following NGF deprivation. This caspase-3 inhibitor is a tetrapeptide analog of the cleavage site of caspase-3. In addition, it appears that the caspase-3 inhibitor effect was specific to the NGF withdrawal mechanism of reactivation. Another known stimulus

of reactivation is a plant derived chemical, forskolin, which is a potent activator of adenylate cyclase. Forskolin initiates an up-regulation of second messenger cAMP (73). The role of forskolin in apoptosis is controversial but in general forskolin does not induce apoptosis in neurons. When forskolin was used to induce reactivation in the previously described latency model, the caspase-3 inhibitor was not effective in attenuating HSV-1 reactivation. Therefore, the data implies that the effect of the caspase-3 inhibitor was specific for interference of the apoptotic pathway of NGF withdrawal suggesting that non-apoptotic pathways can produce reactivation of latent HSV-1 that are caspase-3 independent. However, caspase-3 inhibition did not inhibit normal productive infection in neurons and contrary to previous reports. Previous experiments indicate that herpes contains anti-caspase-3 activity but these experiments were performed with actively dividing cell lines (27). Since neurons are post-mitotic cells, we cannot extrapolate conclusions based on experiments from dividing cell lines. These experiments suggest that caspase-3 induced reactivation of latent HSV-1. Because caspase-3 is a proteolytic enzyme that localizes to the nucleus upon activation, it may be causing reactivation either directly or indirectly.

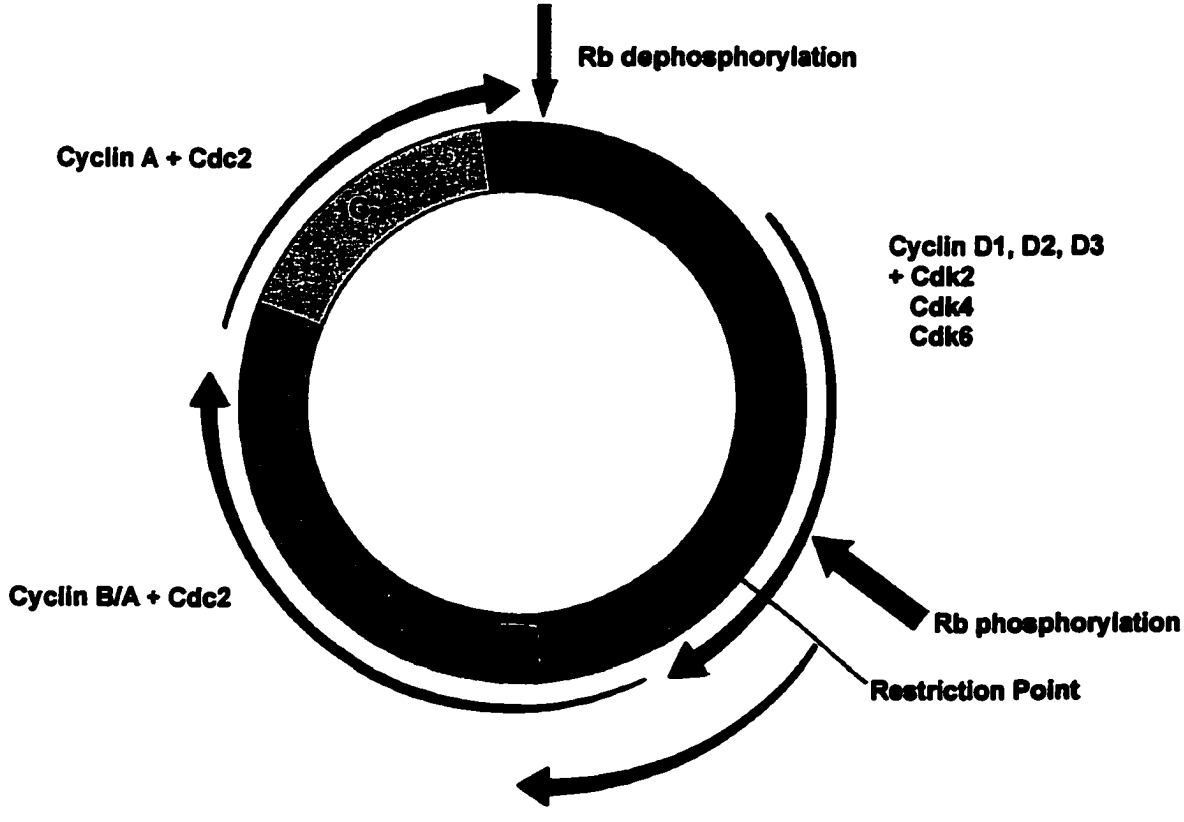
In summary, NGF withdrawal may involve the activation and signaling through the NGF p75 receptor. This signaling may begin the cascade of caspase activation, which in turn cleaves DNA repair enzymes and begins the ultimate cell destruction and suicide. Upon activation, caspase-3 may cleave important viral repressor proteins, such as ICP4, and signal HSV-1 reactivation in order for the virus to begin productive infection to escape its demise. Hence, HSV-1 reactivation may involve direct effects of caspase-3. In addition, caspase-3 may mediate a downstream event to initiate IE gene transcription by

altering intracellular levels of ICP4 or ICP27. This event may progress to either the inactivation of a repressor protein and induction of productive infection. Initiation of these two events, either in combination or separately, may begin the cycle of HSV-1 productive infection and viral spread and survival within its host.

5.2d The Role of Cyclin D1 in HSV-1 Reactivation

In normally dividing cells, the response to differing exterior stimuli determines the ultimate fate of the cell. Often, the presence of growth factor drives the cell into mitosis. The cell cycle is composed of four main phases: G1, S, G2 and M. A change in the exterior environment, such as a growth stimulus, signals the entry into G1 phase, where the cell gears up for DNA synthesis. Progression into the S-phase, where DNA synthesis occurs, involves particular expression of proteins referred to as the cyclins (Fig. 1.5).

Fig. 1.5 Proteins involved in cell cycle regulation (24)



The cyclins normally form complexes with cyclin dependent kinases (CDK) to form a holoenzyme (cyclin-CDK). The cyclins are important regulators of the CDK and determine appropriate target proteins for phosphorylation. Their function can be diverse and include DNA binding or phorphorylation of the retinoblastoma protein (Rb). The assembly, break down and activation of these cyclin-CDK complexes determine the progression or the abortion of the cell cycle.

During cellular division, the nuclear environment changes dramatically. For example, during S-phase, when DNA synthesis begins, the nucleus contains all the transcriptional, metabolic, enzymatic and structural materials necessary for DNA synthesis. Viruses often take advantage of available host cell proteins during different phases of the cell cycle for their own replication. These host cell building blocks, such as amino acids and nucleotides, are more readily available during particular stages of the cell cycle. Therefore, certain viruses have adapted efficient replication during specific phases of the cell cycle. For example, parvoviruses require the progression from G1 to S-phase, where it replicates efficiently. The adenoviruses accelerate the rate of the cell cycle by expressing the E1A protein that physically associates with the retinoblastoma (Rb) protein releasing E2F and driving the cell from G1 into S-phase (3).

In contrast, HSV-1 is a cell-cycle-independent virus and replicates efficiently in post-mitotic cells, such as neurons. In fact, HSV-1 actually halts the cell cycle in normally dividing cells according to studies by Ehmann et al., 2000 (19). This research indicated that HSV-1 stops cells from entering S-phase therefore, preventing host DNA synthesis and not viral DNA synthesis. Some of the essential cell cycle proteins involved in the progression from G1 to S phase include cyclin-D1.

Cyclin-D1 is crucial for the initiation of cell division in mammalian cells however its role in post-mitotic cells is unclear. In 1994, Freeman et al. first described both the transcriptional regulation of the cyclins and their role in sensory neurons (26). Since neurons are terminally differentiated/postmitotic cells, the discovery of these proteins in neurons was perplexing. To date, the function of cyclins besides cell division is unknown. Freeman and colleagues hypothesize that increased expression of cyclins following a positive growth stimulus for cell division initiates apoptosis in neurons. This response ensures that the neuron remains a post-mitotic cell. Their data also indicated that NGF deprivation of sensory neurons increased RNA levels of cyclin D1. In summary, increases in cyclins necessary for cell cycle progression causes apoptosis in neurons.

Cyclin-D1 nuclear increases or decreases are often dependent on Ras activation. Ras protein is a GTPase adaptor molecule normally co-localized with most growth factor receptors. Once the growth factor receptor binds its ligand, Ras is activated and released from the receptor complex. Ras activation initiates a PI-3K increase, which causes the phosphorylation of cyclin-D1 and subsequent ubiquitination. Cyclin-D1 is exported out of the nucleus into the cytoplasm for further degradation by cellular proteosomes. Ras activation can also maintain increased nuclear levels of cyclin-D1 by some unknown mechanism.

In the nervous system perhaps both pathways are actually functioning in parallel maintaining a basal level expression of cyclin-D1 incapable of initiating cell division but essential for normal cellular function. If either pathway is disrupted, then increased nuclear levels of cyclin-D1 may initiate an apoptotic cell death. Since Ras is an adaptor molecule common to most growth factor receptors, it is possible that NGF deprivation

changes Ras signaling. This change may ultimately allow the accumulation of cyclin-D1 in the nucleus, signaling the neuron to initiate the cell cycle. In response to cyclin-D1 accumulation, the neuron undergoes programmed cell death. Our experiments attempt to determine the role of cyclin-D1 in HSV-1 reactivation and apoptosis in the DRG cultures.

6. Potential Convergent Pathways for HSV-1 Reactivation

There may be a common pathway that funnels all the different stimuli for reactivation to a common event of hyperexcitation due to modulation of the nociceptor neuron. Modulation of the nociceptor implies changes in ion channel expression and resting membrane potential of the neuron. These changes often reduce the strength of the stimulus required for depolarization in order to elicit an action potential. This neuronal modulation or sensitization occurs following exposure to prostaglandins, epinephrine and NGF deprivation that happens during tissue damage following herpetic lesions. Previous characterization of the signal transduction pathways involved in HSV-1 reactivation uncovered the potent effects of forskolin and a phorbol ester compound, PMA (73). Stimulation of latently infected DRG cultures with these compounds induced reactivation. Additionally, our studies showed that treatment of HSV-1 latently infected neurons with capsaicin or heat shock also induced reactivation. The common theme among all of these stimuli is hyper-excitation of the nociceptor neuron leading to neuronal modulation. This change in neuronal plasticity may explain the experience of severe pain and hyperalgesia during herpes eruptions on the epithelial surface (81).

The common factor that ties these seemingly unrelated stimuli for reactivation to hyperexcitation of the nociceptor neuron may involve activation of the calcium ion

channel, VR-1. For example, forskolin induces up-regulation of protein kinase A (PKA), which is known to activate the VR-1 receptor (50). PMA activates the second messenger protein kinase C (PKC), which stimulates sensory neuron specific sodium channels (SNS) and enhances VR-1 activation to excite the neuron (14, 41, 65, 83). Furthermore, heat and capsaicin stimulation induces excitation of VR-1, causing increases in intracellular calcium leading to depolarization and firing of the neuron (60). All of these events include some aspect of excitation of the nociceptor neuron. Finally, the in vitro latency model was initially characterized using a neuronal stress stimulus of NGF deprivation to induce reactivation of the latently infected neurons (86). NGF deprivation could involve activation of the low affinity NGF receptor, p75, which is also believed to modulate VR-1 activity and is thought to be important in pain (70, 80). Additionally, NGF regulates intracellular calcium levels in the neuron via IP-3K.

7. Summary

In conclusion, the VR-1 may play a key role in both HSV-1 primary infection and establishment of latency and reactivation. Based on our data, stimulation of VR-1 induces reactivation. Conversely, inhibition of this receptor blocks heat shock and forskolin induced HSV-1 reactivation. These results support the hypothesis that herpes reactivation is dependent on hyperexcitation of a nociceptor induced by VR-1 activation. Additionally, most of the research characterizing HSV-1 has not been done in sensory neurons and perhaps this is the reason why the role of VR-1 relative to HSV-1 has never been reported. These studies may reveal a novel model for reactivation that provides

clues to the human condition of fever-induced reactivation for future anti-viral drug designs.

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Chapter 2

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Caspase-3-Dependent Reactivation of Latent HSV-1 in Sensory Neuronal Cultures

Abstract

Life-long latent herpes simplex virus type 1 (HSV-1) infections are common and affect over 60-80 % of the general population. Latent virus is harbored in sensory neurons of the peripheral nervous system. Sporadic reactivation of latent HSV-1 is associated with a number of apparently unrelated stimuli, including stress, fever and trauma to the skin. These stress factors may involve apoptotic signaling in neuronal cell bodies. Our studies suggest that reactivation of latent HSV-1 following nerve growth factor (NGF) deprivation stimulated apoptotic signaling by activating the pro-apoptotic proteolytic

enzyme, caspase-3. In dorsal root ganglion (DRG) neuronal cultures harboring latent HSV-1, treatment with a caspase-3 specific inhibitor significantly reduced NGF deprivation-induced reactivation. However, the caspase-3 inhibitor did not prevent reactivation induced by stimuli previously shown to induce reactivation, including forskolin and phorbol 12-myristate 13-acetate (PMA), activators of protein kinase A (PKA) and protein kinase C (PKC), respectively. Interestingly, treatment with the caspase-3 inhibitor had no effect on productive HSV-1 infection in the neuronal cultures. Our data indicate that caspase-3 is involved in signaling following NGF deprivation-induced reactivation of latent HSV-1. Activation of caspase-3 using staurosporine did not cause viral reactivation in the cultures. However, activation of caspase-3 with C2-ceramide caused significant reactivation. Furthermore, direct effects of caspase-3 using a recombinant adenovirus over-expressing caspase-3 induced reactivation of HSV-1 latently infected cultures. These data suggest a direct role of caspase-3 in reactivation of latent HSV-1 in neurons.

Introduction

Herpes simplex virus type 1 (HSV-1) infections normally manifest as fever blisters on mucous membranes or skin (reviewed by (2)). A more severe effect of the virus includes infection of the corneal epithelium, the leading infectious cause of blindness (8). Furthermore, HSV-1 infection in the brain results in encephalitis, a serious condition leading to mortality of the infected individual. The recurrence of herpes infections is mostly attributed to reactivation of the latent life-long persistent infection (27). The most intriguing aspect of HSV-1 is unraveling the mystery of latency within the sensory

neuron cell bodies of the dorsal root and trigeminal ganglion cells. The sensory neurons are the primary site of HSV-1 latency in humans. (25).

The virus gains access to nerve termini during the productive infection of mucocutaneous membranes. HSV-1 is retrogradedly transported via the axon to the neuronal cell body to establish a latent infection (3). Following various stimuli, the virus can sporadically reactivate. Upon reactivation, HSV-1 is antegradedly transported back to re-infect the dermis to repeat the cycle of infection. To date there is no known pharmacological agent capable of preventing or eliminating HSV-1 latency.

We have developed an *in vitro* model to study HSV-1 latency and reactivation in sensory neurons in cultures (28). This *in vitro* neuronal model recapitulates HSV-1 latency in humans and animal models (9, 28, 30). In this model, reactivation is induced by various stress stimuli including nerve growth factor (NGF) deprivation (29).

Often trophic factors play an important role for neuronal survival. In the developing nervous system, NGF is an important neurotrophic factor necessary for the development, growth, survival, maintenance and repair of the sensory neurons (12). These effects are mediated through two receptors that bind NGF. The TrkA (tyrosine kinase A) or p140 receptor is essential for cell survival and the NGF p75 receptor appears to signal apoptosis (4, 13, 15, 19). *In vitro*, NGF deprivation often induces an apoptotic signal transduction pathway in sensory neurons (Reviewed in (16). The mechanism of apoptosis following NGF deprivation is complex and may involve the lack of signaling of the TrkA and/or the initiation of p75 signaling.

The NGF p75 receptor is in the same receptor family as the tumor necrosis factor-alpha (TNF-α) R1 and the Fas/Apo-1 receptors, suggesting that these receptors share

similar apoptotic signaling (22). Both the TNF-αR1 and Fas/Apo-1 receptors contain a cytoplasmic death domain region capable of binding adaptor molecules, such as caspase-8 (5). The cysteinyl aspartate-specific proteases (caspases) are characterized as important activators of the apoptotic signal transduction pathway, possibly via the NGF p75 receptor following NGF deprivation.

Activation of the caspases initiates an enzymatic cascade resulting in apoptosis (reviewed by (18)). Apoptosis is a neat and orderly process where cell death begins as sequential enzymatic reactions ultimately ending in the fragmentation of DNA and condensation of the cell. This process is especially important in the developing nervous system and necessary for proper neuronal connections. The importance of apoptosis in the nervous system became evident following the severely altered neuronal development in the caspase-3 knock-out mouse. These animals developed enlarged nervous systems and were severely compromised by the lack of normal developmental neuronal apoptosis (17).

Caspase-3 is activated from the pro-enzyme form by caspase-8. Once proteolytic cleavage of caspase-3 occurs, the activated enzyme translocates to the nucleus to cleave its substrate target proteins including the DNA repair enzyme, Poly (ADP ribose) Polymerase (PARP) and DNA-protein kinase (DNA-PK). The ultimate downstream events following caspase-3 activation include DNA fragmentation and chromatin condensation, which are hallmarks of the end stages of apoptosis. Our studies focus on the apoptotic signaling pathways and the relevance of caspase-3 activation for initiating HSV-1 reactivation following a latent infection.

Materials and Methods

Cell culture. Vero cells obtained from ATCC (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine serum (Life Technologies, Rockville, MD). Sensory neuron cultures were prepared from DRG of embryonic day 15 Sprague-Dawley rats as previously described (24, 30). Neurons were plated onto 24 well plates at a cell density of 1-5 $\times 10^3$ cells per well. Dulbecco's Modified Eagle's/F12 medium supplemented with 10% newborn bovine serum and 100 ng/ml 2.5 S nerve growth factor (Harlan Bioproducts Indianapolis, IN) was used to maintain neuronal cultures (neuronal maintenance medium). Cultures were treated with fluorodeoxyuridine (20 μM) for 7-10 days after plating to reduce the non-neuronal cell population.

Establishment of latent HSV-1 infections. Latent HSV-1 infections in neuronal cultures were established as previously described (24, 28, 30). After neurons were established in tissue culture for 2 weeks, 50μM acycloguanosine (Sigma, St. Louis, MO) was added to the culture medium 24 hours prior to inoculation with virus and for the following 7 days after inoculation. Neuronal cultures were infected with approximately 10 plaque-forming units (PFU) of HSV-1 (17^+) per neuron.

Reactivation of latent HSV-1. The cultures were maintained latently infected for 14 days prior to reactivation. Reactivation was induced by: (1) NGF withdrawal and subsequent treatment with anti-NGF antibodies for 5 days (2) Forskolin (Sigma, St. Louis, MO) treatment at 100μM for 3 days (3) PMA (Sigma, St. Louis, MO) treatment at

10^{-10} M for 3 days (4) C2-ceramide at 25μM and 50μM for 3 days and (5) Staurosporine at 100nM and 250nM for 1 and 5 days. The neuronal cultures harboring latent HSV-1 were treated with anti-NGF, PMA or forskolin and concomitantly with the cell permeable caspase-3 specific inhibitor, DEVD-CHO (Calbiochem, La Jolla, CA.). Reactivation was determined using a standard viral plaque assays. Briefly, Vero cells were plated on 24-well plates and incubated with aliquots of cell extract from DRG latently infected cells. Plaque formation occurred within 48 hours post infection. The plaques were counted to determine viral titers for each well. Each experiment consisted of an n of 6 for each treatment group and was repeated three to five times. The data was analyzed using a one-way ANOVA using the SAS statistical software.

Productive Infection. In order to establish a productive infection in the DRG neurons opposed to a latent infection, the cells were infected at a higher MOI of 50 pfu per neuron for one hour. The virus was removed and the cells were washed and incubated for 24 hours following initial infection. The DRG neurons were pre-treated with 1, 10 and 100nM caspase-3 inhibitor, DEVD-CHO (Calbiochem, La Jolla, CA.) for 2 hours prior to the initial infection and re-treated following removal of virus and wash. The viral titers were determined using a standard plaque assay as previously described.

Construction of recombinant adenovirus: pET-23b plasmid backbone containing human CPP32 complete coding sequence was purchased from ATCC (Cat. # 99625, PubMed 96355448). CPP32 was excised from the pET-23b using NdeI and XhoI and sub-cloned into the left end of the adenovirus plasmid (E1A promoter) to create pE1A-

CPP32 (8). Both the pE1A-CPP32 and pJM17 were co-transfected into 293 cells to generate the recombinant adenoviral vector, Ad-E1A-CPP32. Adenovirus viral titers are obtained using a standard plaque assay. MOI of approximately 50 or 100 were used to infect the neurons. Previous experiments using AdE1A-EGFP virus as a control did not cause HSV-1 reactivation (7). Following infection with Ad-E1A-CPP32 over-expressing caspase-3, the neurons were harvested 5 days later. HSV-1 viral titers were determined using a standard plaque assay as previously described.

Results

Caspase-3 inhibitor attenuates HSV-1 reactivation following NGF deprivation but does not prevent reactivation induced by forskolin or PMA treatment.

In order to determine the role of caspase-3 in HSV-1 reactivation, we treated the neurons with a tetrapeptide analog inhibitor of caspase-3, DEVD-CHO, prior to NGF deprivation. This inhibitor is a specific tetrapeptide analog of the cleavage site of caspase-3, thus the enzyme cannot be cleaved to its active form. The caspase-3 inhibitor induced a 2-fold reduction in viral titers following HSV-1 reactivation following NGF deprivation (Fig 2.1). Based on NGF withdrawal experiments, the data suggest that caspase-3 activation is important for HSV-1 reactivation induced by NGF deprivation.

Previous data indicated that activation of protein kinase C with PMA or activation of protein kinase A using forskolin induced reactivation of HSV-1 in latently infected neurons (23). The caspase-3 inhibitor was tested to determine if caspase-3 was also involved in reactivation induced by PMA and forskolin. The data showed that the

inhibitor was not effective in attenuating HSV-1 reactivation following forskolin treatment. Similar results were observed following PMA-induced HSV-1 reactivation showing that the caspase-3 inhibition did not prevent reactivation (Fig. 2.2). It appears that the caspase-3 inhibitor effect was specific to the NGF withdrawal mechanism of reactivation.

Caspase-3 inhibitor has no effect on productive HSV-1 infection.

To determine whether caspase-3 inhibitor was specific to reactivation of latent HSV-1, the caspase-3 inhibitor was tested following a productive infection in the DRG cultures. The cultures were treated with the caspase-3 inhibitor for one hour prior to infection and for an additional 24 hours after the initial infection. HSV-1 viral titers were used to assess the effects of the inhibitor. The data indicated no statistical significance between the caspase-3 inhibitor and the untreated controls (Fig. 2.3). The results show that the caspase-3 inhibitor had no effect on HSV-1 replication in DRG neurons.

Activation of caspase-3 by C2-ceramide induced reactivation of latent HSV-1.

Two known intracellular activators of caspase-3 differed in their ability to induce HSV-1 reactivation from latently infected cultures. C2-ceramide, a product of sphingomyelin hydrolysis by sphingomyelinase activation, induced significant dose-dependent reactivation (Fig. 2.4). Conversely, the antibiotic compound, staurosporine used at concentrations of 100, 250 and 500nM and time points varying from 1-5 days did not induce HSV-1 reactivation in the *in vitro* neuronal model of HSV-1 latency (Table 2.1).

The recombinant adenovirus over-expressing caspase-3 induced reactivation in HSV-1 latently infected neurons.

In order to examine the direct effects of caspase-3 on HSV-1 reactivation, an adenovirus vector was used to over-express caspase-3 because DRG neurons are difficult to efficiently transfect. Adenovirus vectors have been used to efficiently introduce and express transgenes in DRG neurons in vitro (7). The caspase-3 over-expressing adenovirus caused reactivation of HSV-1 latently infected neuronal cultures at the two different MOI tested (Fig. 2.5).

Discussion

HSV-1 alters apoptotic pathways during a productive infection in vitro in many different cell lines, but the effects of HSV-1 on apoptosis during a latent infection in neurons has not been elucidated (1, 10, 14, 20, 31). Previously, our laboratory demonstrated that NGF deprivation initiated HSV-1 reactivation from latently infected DRG neurons in culture (28). NGF deprivation initiates an apoptotic-signaling pathway in immature neurons often dependent on caspase-3 (reviewed by (16). In order to determine the mechanism essential for NGF deprivation-induced reactivation of latent HSV-1, we used a specific inhibitor to caspase-3. Based on our results with the caspase-3 inhibitor, we determined that caspase-3 plays an essential role in NGF deprivation induced HSV-1 reactivation. However, the caspase-3 inhibitor could not completely abolish viral reactivation, suggesting that other pathways must also be involved. Since NGF binds two important receptors in the nervous system that activate and deactivate many different cellular pathways, caspase-3 may play a partial role in the NGF-induced apoptosis pathway.

Also, our DRG cultured neurons were allowed to mature in culture for 14 days, which alters their dependence on NGF for survival and could explain the partial protection provided by the caspase-3 inhibitor (26).

Activation of caspase-3 following NGF withdrawal may involve signaling through the NGF p75 receptor. If the NGF p75 receptor behaves as an NGF sensor, determining the available amount of NGF present for the cell, we postulate that the NGF p75 receptor may contain adaptor molecules that are directly linked to the activation of the caspases. Since the NGF p75 receptor belongs to the same family of receptors as the TNF-α and Fas/Apo-1, it may contain adaptor molecules such as caspase-8. Activation of the caspases may be mediated through a putative death domain on the cytoplasmic region of the NGF p75 receptor. The lack of binding of NGF to the NGF p75 receptor after NGF withdrawal may initiate proteolytic activation of the caspases (21). This signaling may begin the cascade of caspase activation, which in turn cleaves DNA repair enzymes and begins the ultimate cell destruction and suicide.

To further understand the involvement of caspase-3, we tested other known activators of caspase-3 *in vitro*, including C2-ceramide and staurosporine. C2-ceramide is generated following the hydrolysis of sphingomyelin by the activation of the enzyme, sphingomylinase. During apoptosis, levels of C2-ceramide correlate with caspase-3 cleavage and activation. The NGF p75 receptor causes C2-ceramide production upon activation, implicating NGF p75 as an important signaling receptor following NGF deprivation (6). By using cell permeable C2-ceramide, we confirmed that the caspase-3 dependent pathway also could induce reactivation of latent HSV-1.

Interestingly, staurosporine did not cause HSV-1 reactivation in the *in vitro* latency model. Staurosporine is an antibiotic compound developed from *Streptomyces staurosporeous* and frequently used to induce caspase-3-dependent apoptosis. However, this result was not surprising, since staurosporine is also a non-specific kinase inhibitor and may have affected other essential viral, as well as host, kinases necessary for viral DNA synthesis. Furthermore, stuarosporine specifically inhibits potent inducers of HSV-1 reactivation, including PKA and PKC. These two kinases act independent of caspase-3, since our experiments indicate that inhibition of caspase-3 following induction of PKA and PKC does not prevent viral reactivation. Nevertheless, the C2-ceramide data suggest a direct effect of caspase-3 in HSV-1 reactivation.

Previously published data indicate that HSV-1 is both dependent and independent on caspase-3 activation during a productive infection (10). Experiments by Munger et al., (2001) implicate viral protein US3 as a caspase-3 inhibitor (20). The data presented here demonstrate that in neurons, caspase-3 inhibitors have no effect on the productive HSV-1 infection. Therefore, the effects of the caspase-3 inhibitor were specific for HSV-1 induced reactivation from a latent infection and only essential for apoptotic signaling pathways. It is possible that caspase-3 is important during a productive infection in non-neuronal cells to maintain an appropriate level of ICP4 and ICP27 that promotes viral gene transcription and not repression. Since ICP4 and ICP27 both have caspase-3 targeted cysteine-aspartate sequences, they may compete with caspase-3 host cellular targets and therefore prolong cell survival and ensure the progression from alpha to beta gene transcription. Mutant studies verify that in the absence of ICP4 and ICP27, the cell is not protected from apoptosis (1, 11). So it is possible that the virus requires the

breakdown of ICP4 and ICP27 and relies on apoptotic enzyme activity for its own purposes.

The data obtained in our experiments implicate apoptotic-signaling pathways as mediators of HSV-1 reactivation. Our experiments show that a specific caspase-3 inhibitor attenuates NGF deprivation-induced HSV-1 reactivation. This appears to be specific for NGF-induced reactivation since the caspase-3 inhibitor did not affect HSV-1 reactivation following treatment with forskolin or PMA; two pharmacological agents known to cause reactivation in this *in vitro* model but do not cause apoptosis (23). Furthermore, the caspase-3 inhibitor does not affect viral titers during HSV-1 productive infection. We conclude that caspase-3 activation has a direct effect on latent HSV-1 because C2-ceramide, an activator of caspase-3 *in vitro*, caused HSV-1 reactivation. Moreover, the recombinant adenovirus over-expressing caspase-3 implicates a direct role of caspase-3 in reactivation of HSV-1 latently infected cultures. Taken together, these experiments show a direct effect of caspase-3 as an important mediator for HSV-1 reactivation following apoptosis signaling

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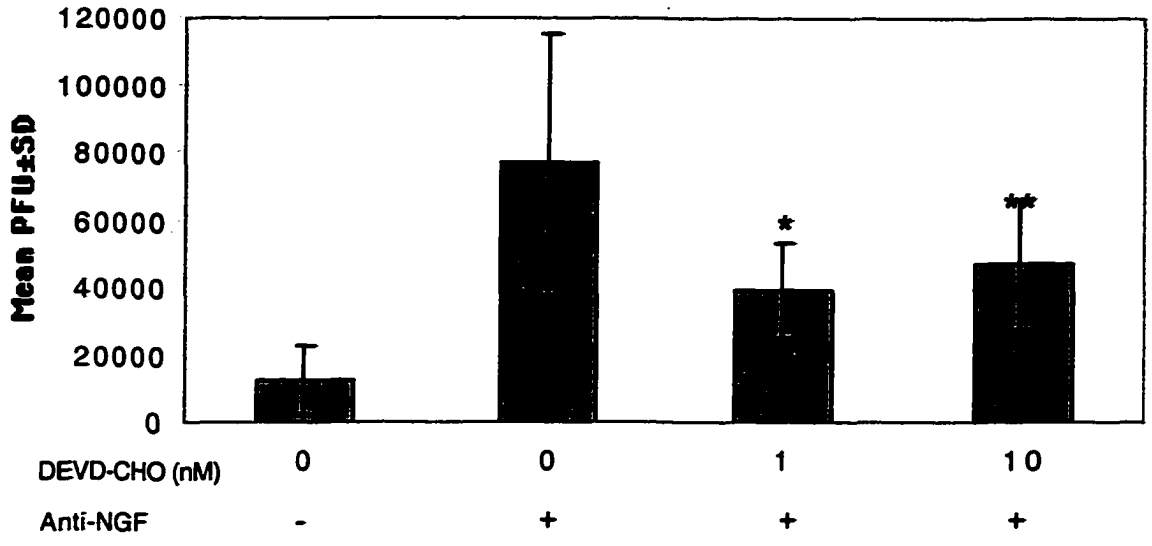

*Figure 2.1 NGF withdrawal induced HSV-1 reactivation in latently infected DRG neurons is attenuated by treatment with caspase-3 inhibitor. DRG cultures were latently infected with an MOI=5 with HSV-1 17+. The cultures remained latent for 14 days. Reactivation of HSV-1 was induced by NGF deprivation. Five days following reactivation, the cultures were harvested for a standard viral plaque assay. Each treatment group consist of n=18. SAS statistical software analysis using a one-way ANOVA with a Tukey-Kramer adjustment determined that at Caspase-3 inhibitor concentrations of 1nM * p=02 and 10nM ** p=.015.*

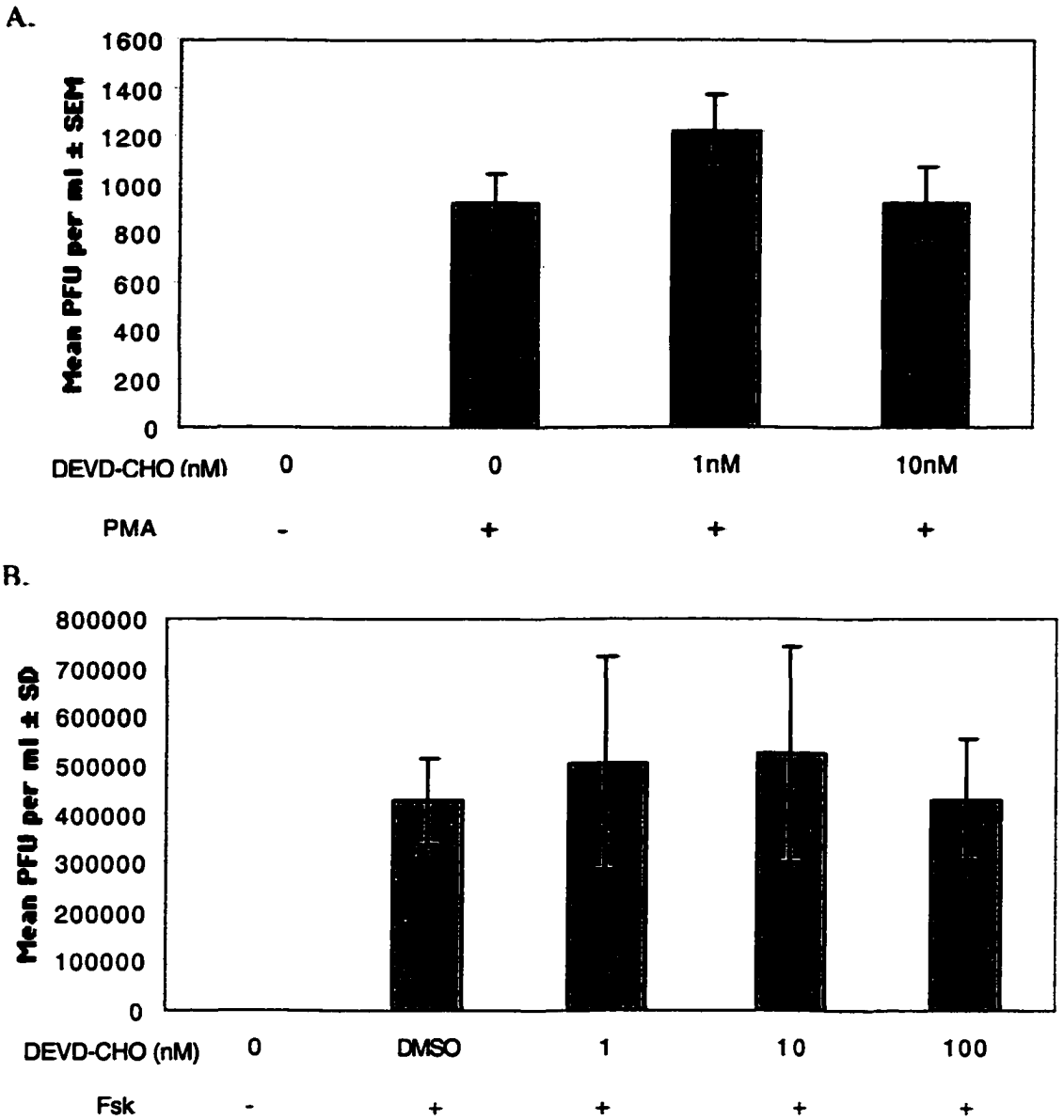

Figure 2.2. The effects of the caspase-3 inhibitor following PMA or forskolin induced HSV-1 reactivation. A. DRG latently infected neurons were pre-treated for 1 hour with caspase-3 inhibitor (DEVD-CHO) prior to reactivation with 100μM forskolin. Each group represents n=6. The data was analyzed using a one-way ANOVA, which determined no significant differences between caspase-3 inhibitor treatment groups. B. The effects of the caspase-3 inhibitor following 1X10-9 M 13-phorbol-12-myrestate acetate (PMA) induced reactivation of latent cultures. Each group represents n=6 and SAS analysis using a one-way ANOVA determined no significant differences between caspase-3 inhibitor

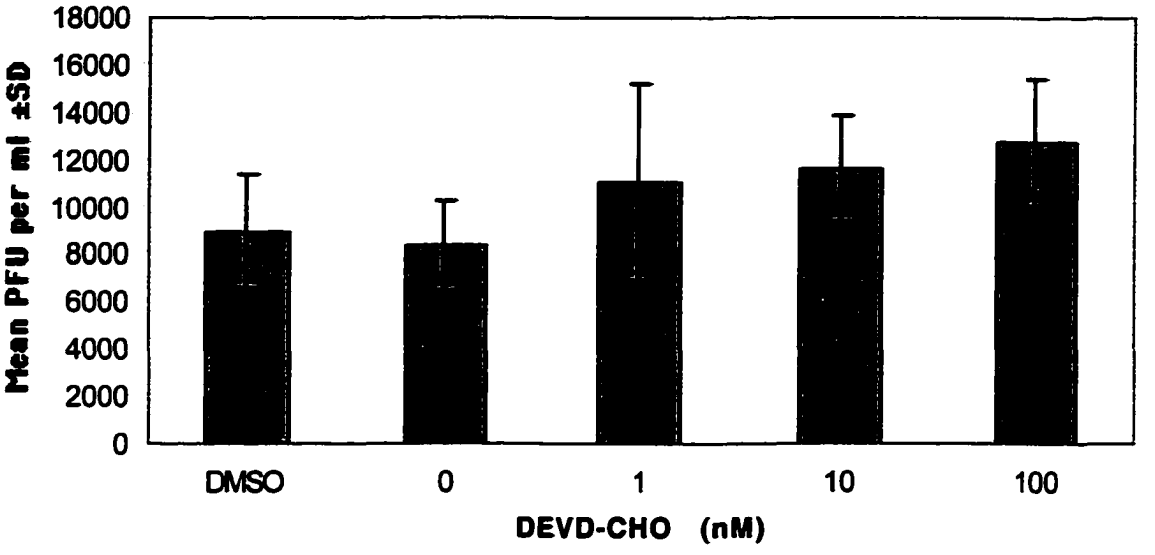

Figure 2.3. Caspase-3 inhibitor did not affect viral replication during a productive infection in DRG cultures. The neurons were pre-treated with the caspase-3 inhibitor and then infected with HSV-1 at an MOI=50. Viral titers were assessed following 24 hours post-infection. One-way ANOVA showed no significant difference between treatment groups. Each group consisted of n=6.

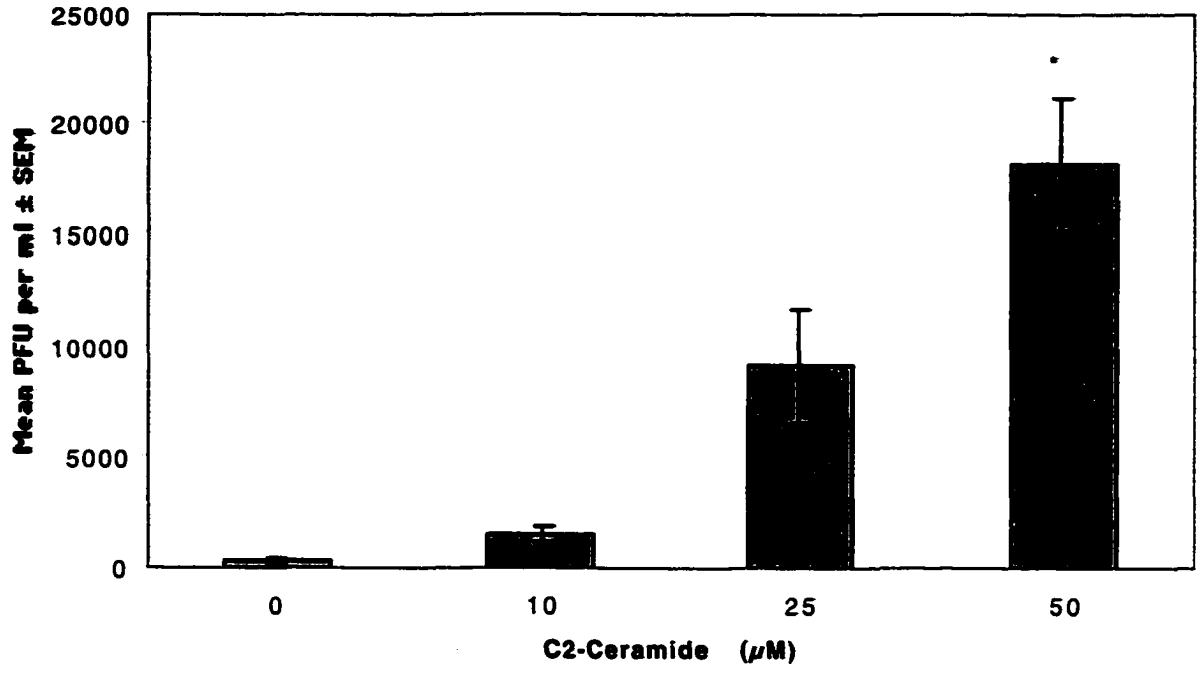

Figure 2.4. C2-ceramide induced HSV-1 reactivation in latently infected DRG cultures. Latent cultures were treated with C2-ceramide for 4 days and subsequently analyzed for infectious HSV-1. The data are representative of 5 separate experiments with n=18 for each treatment group. Analysis using an ANOVA determined that at 50μM C2-ceramide was statistically significant from control cultures (p=.0054).

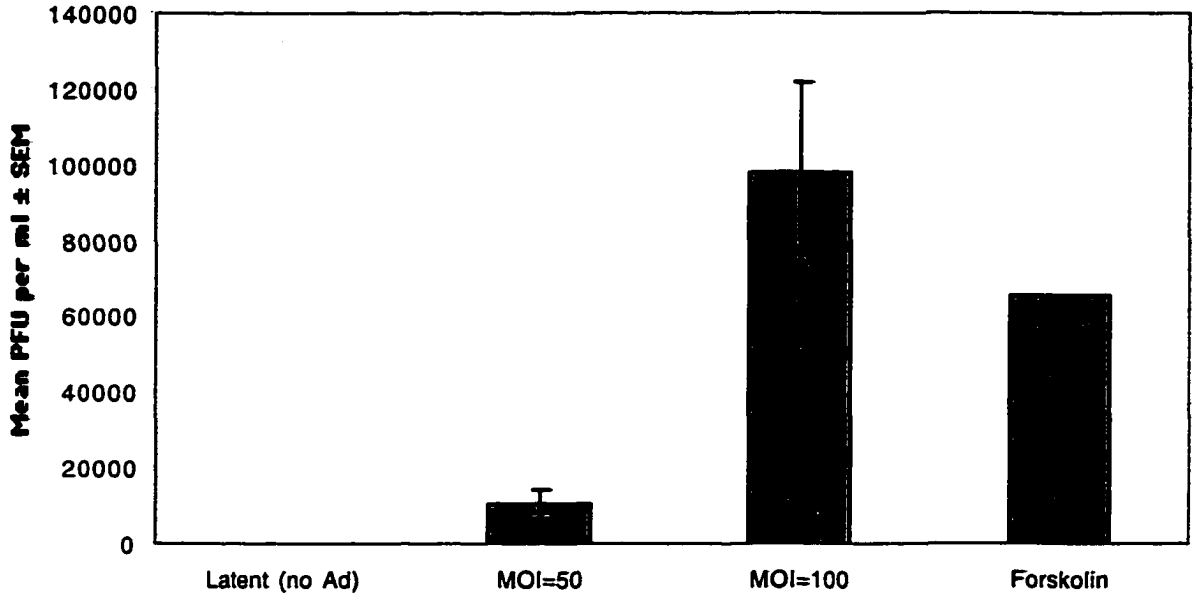

Figure 2.5. Recombinant adenovirus overexpressing caspase-3 (Ad-E1A-CPP32) induced an MOI-dependent HSV-1 reactivation in latently infected DRG cultures. Control latent cultures were not treated with adenovirus. Positive control of 100μM forskolin was used to show that latent HSV-1 was present. Previous studies show that the adenovirus vector backbone has no effect on latently infected DRG neurons in culture (8).

Staurosporine (nM)	HSV-1 Reactivation (days)	Effect
500nM	20hrs	none
250nM	20hrs	none
250nM	3 days	none
100nM	3 days	none
500nM	2 days	none
250nM	5 days	none

Table 2.1. Effect of staurosporine of latently infected DRG neurons. HSV-1 latently infected DRG cultures were treated with staurosporine at the given concentrations. The data indicated that staurosporine did not cause reactivation. Each treatment group consisted of n=6.

Appendix to Chapter 2

The results discussed in this section were not included with the manuscript. Some of the data are peripheral to the chapter and include interesting studies that I could not fully pursue due to time limitations. The data discuss anti-apoptotic mechanisms of LAT protein, caspase-3 RNA data from Millenium Pharmaceuticals and mechanistic studies of the role of caspase-3 in HSV-1 reactivation.

LAT as an anti-apoptotic protein

Introduction

Previous report by Perng et al., 2000 showed that LAT had anti-apoptotic activity. These studies mostly focused on the LAT transcript and not the putative protein product of this transcript. In order to determine whether the putative LAT protein product from open reading frame 1 (ORF-1) had anti-apoptotic effects, we used a PC12 apoptotic model (6). This adrenal pheochromocytoma cell line can be differentiated to sensory neuron-like cells with NGF. Following differentiation, PC12 are dependent on NGF for their survival and undergo classic apoptosis in the absence of NGF.

Materials and Methods

In order to study the effects of the LAT protein a recombinant adenovirus expressing LAT Open Reading Frame-1 (ORF-1) was developed by Mark Colgin. This recombinant

adenovirus was tested in a classic PC12 apoptosis model using NGF deprivation. Briefly, undifferentiated PC12 cells were plated on collagen coated 6 well plates and differentiated for seven days with 50μg/ml of NGF in RPMI 1640 medium containing 10% heat-inactivated horse serum and 5% fetal bovine serum (Gibco BRL). Differentiated cells possess neuronal-like morphology including neurite projections following NGF treatment. To induce apoptosis NGF is withdrawn from the medium. Prior to NGF deprivation the differentiated PC12 cells were infected with the recombinant adenovirus over-expressing LAT-ORF-1. The cells were visually analyzed daily and an Almar Blue assay was performed as previously described in Chapter 2 to determine cell viability. There were three wells per treatment group and the treatment groups included: 1) NGF deprived PC12 cells 2) Ad-ORF-1 infected NGF deprived PC12 cells 3) Ad-GFP infected NGF-deprived PC12 cells.

Results and Discussion

Our results indicated that the putative LAT protein had no anti-apoptotic effects in PC12 cells. Following NGF withdrawal, the differentiated PC12 cells died despite the presence of the LAT protein (Fig.2.6). The experiment implied that there was accelerated cell death in the presence of Ad-ORF-1 compared to PC12 NGF deprived alone. Although this datum indicates that expression LAT-ORF-1 was unable to promote PC12 survival in the absence of NGF, it does not account for the possible anti-apoptotic effects of the LAT RNA transcript itself.

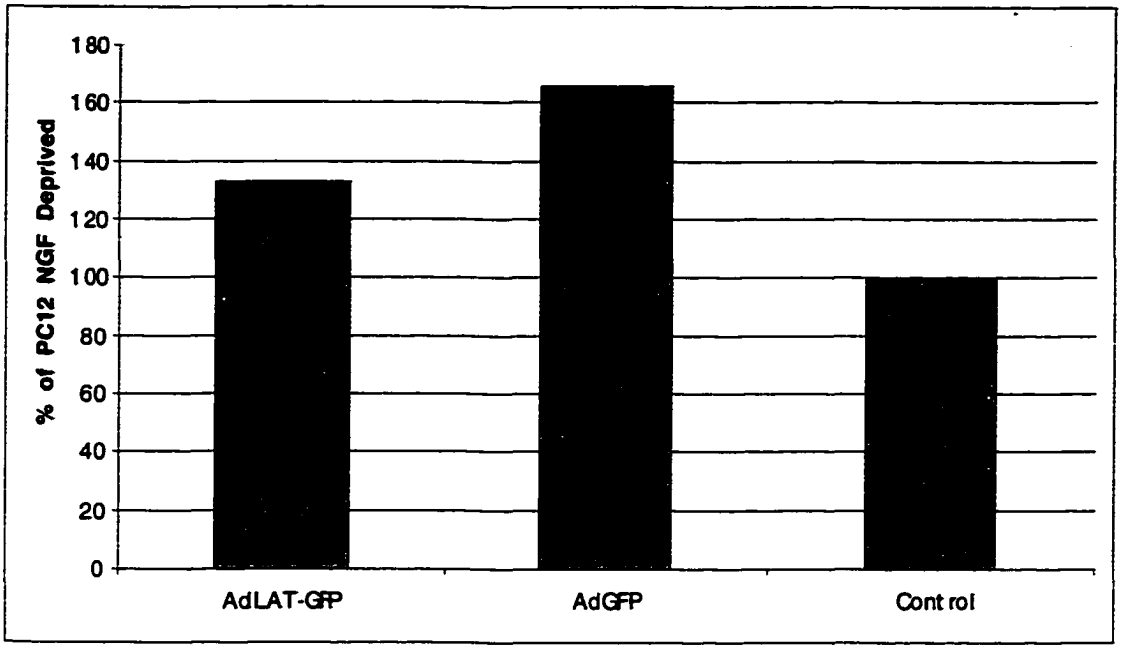

Figure 2.6 PC-12 cell survival assay in the presence of LAT protein expression

LAT protein expression in mature vs. immature neurons

Introduction

The LAT transcript has never been studied in the developing nervous system. Because neonates response to a primary HSV-1 infection can sometimes be fatal and often results in neurological impairments following the primary infection, we conjectured that the differences in a developing nervous system was important for viral replication. We initially noted that there was altered expression of LAT protein in differentiated and undifferentiated PC12 cells and later discovered that the same effect occurred in mature and immature DRG neurons. These findings may elucidate the reason behind the severe ramifications of a herpes infection in neonates compared to adult humans.

Materials and Methods

PC12 cells were differentiated as previously described. Differentiated cells were infected with Ad-ORF-1-GFP or Ad-GFP for 10 days. The cells were visually followed for 10 days and we noted the time period necessary for GFP expression following infection with the recombinant adenoviruses and the disappearance of GFP expression. The same experiment was performed on DRG neurons. E-15 DRG neurons were allowed to mature in culture for 21 days following initial plating versus immature neurons were treated with the recombinant adenoviruses following plating with no maturation period in culture.

Results and Discussion

Both undifferentiated PC12 cells and immature DRG neurons had a delay in Ad-ORF-1 expression following infection. Undifferentiated PC12 cells that were infected with the recombinant adenovirus expressing LAT-ORF-1 fused to GFP displayed a four-day delay in the expression of the GFP fused protein following infection. Conversely, differentiated PC12 cells expressed the LAT-ORF-1-GFP protein within 24-hours following the initial infection with the recombinant adenovirus. We concluded that efficient LAT protein expression required terminally differentiated PC12 cells.

The same results were observed in young neurons (E15 rat DRG) versus mature neurons (E15 rat DRG matured in culture for 21 days). When young neurons were infected with the recombinant adenovirus LAT-ORF-1-GFP, expression was suppressed and we did not observe any GFP expression 24-hours following the initial adenovirus infection. As the neurons matured in culture, the LAT protein fused to GFP was then

expressed hence the PC12 results correlated with the observations obtained in the DRG neurons.

Two important conclusions can be formed from this data. First, it indicates that neuronal maturation affects the expression of LAT, which may be crucial for the establishment of latency and reactivation. These data may also provide an explanation regarding differences in HSV-1 virulence in neonates versus adult humans. Perhaps LAT expression is important for latency and in the absence of LAT a productive infection ensues in neonates instead of a latent infection. Second, non-terminally differentiated cells repress LAT protein expression. This putative repression that is observed in both dividing cells and immature neurons might explain the unique abundance of LAT transcript expression in neurons and the lack of the LAT transcript in normally dividing cells. Nevertheless, our anti-apoptotic data are contradictory to those of other investigators because the LAT protein does not display any anti-apoptotic activity in our assay system. Yet, our data is based on the assumption that the LAT protein is expressed and does not rule out the fact that the LAT transcript itself may have anti-apoptotic activity (3).

Caspase-3 RNA levels increase following NGF deprivation

Real Time RT-PCR analysis of total RNA from HSV-1 latently infected DRG cultures deprived of NGF for 1 and 3 hours indicated a 17-fold increase in caspase-3 mRNA. This analysis was performed by Millenium Pharmaceuticals (Boston, MA) and further validates the caspase-3 results in Chapter 2 (Fig. 2.6).

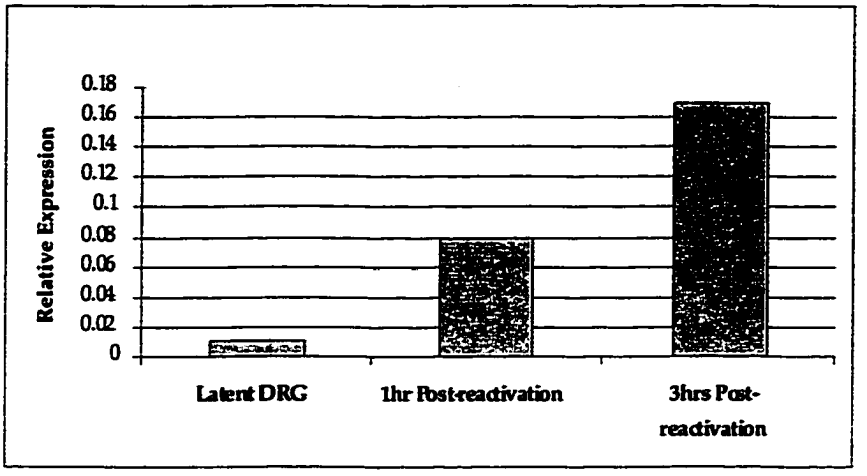

Figure 2.7. Taqman analysis of caspase-3 RNA relative expression following NGF deprivation induced HSV-1 reactivation determined from total DRG RNA

Caspase-3-mediated ICP4 proteolysis

Introduction

The presence of ICP4 in latently infected neurons has been a topic of debate for many years. Margolis et al., believe that spontaneous reactivation occurs in the ganglia and may explain the presence of ICP4 in latent infections (2). Despite the presence or absence of ICP4 during latency, ICP4 can be cleaved by caspase-3. Perhaps cleavage of ICP4 by caspase-3 is more relevant in a productive infection versus a latent infection. As previously discussed in Chapter 2, computer analysis verified caspase-3 cleavage sites within two different HSV-1 immediate early proteins. ICP4 and ICP27 both have cleavage sites within the protein sequence that correspond to caspase-3 target sequences. Productive infection experiments performed in Vero cells confirmed that ICP4 underwent proteolysis following caspase-3 activation with stuarosporine treatment but ICP27 did not. These experiments may provide evidence for a novel anti-apoptotic mechanism used by HSV-1 during a productive infection.

Materials and Methods

Vero cells were infected with HSV-1 at a multiplicity of infection (MOI) of 10 for one hour. The cells are washed after the one-hour incubation with HSV-1 17+ and subsequently treated with staurosporine at 1μM for 4 hours. During a productive infection maximum ICP4 and ICP27 expression occurs normally within four hours following infection. The cell extract was harvested and run on a 12% polyacrylamide gel (SDS-PAGE). The protein was transferred to a nitrocellulose membrane and immunoblotted with a polyclonal antibody to ICP4 and ICP27. The secondary IgG mouse antibody was conjugated to horseradishperoxidase (Vector Laboratories Inc., Burlingame, CA). We used the standard chemiluminescent substrate (NEN Life Science Products, Inc., Boston, MA) and exposed the nitrocellulose membrane to Kodak Biomax ML film to visualize the protein bands (Fig.2.7A and 2.7B).

Results and Discussion

The presence of ICP4 transcripts have been reported in latently infected trigeminal ganglia (1, 4). Furthermore, some studies have shown that ICP4 mutants are unable to reactivate (5, 7). This implies that ICP4 is involved in the maintenance of latency and possibly the induction of reactivation. Caspase-3 activation and translocation to the nucleus may target not only its host substrate but also ICP4. This proteolysis may release the viral genome from repression and initiate a lytic infection. The data showed that although ICP27 was not affected by treatment with staurosporine, ICP4 showed possible

proteolysis. This proteolysis was caspase-3 dependent because pre-treatment with the caspase-3 inhibitor at 10nM decreased proteolysis of ICP4 (Fig. 2.7A).

Additionally, if caspase-3 targets ICP4 then we could speculate that caspase-3 may be important during a productive infection in normally dividing cells. HSV-1 may utilize caspase-3 for two reasons. First, HSV-1 may interfere and delay apoptosis in normally dividing cells by interfering with caspase-3 normal cellular target. Therefore, caspase-3 is cleaving HSV-1 proteins instead of the host nuclear pro-apoptotic proteins such as PARP and DNA-PK. Furthermore HSV-1 may require ICP4 proteolysis to transition the replication cycle from alpha gene expression to beta gene expression. Although further studies are required to confirm this hypothesis, preliminary data along with other published studies strongly suggest this is so.

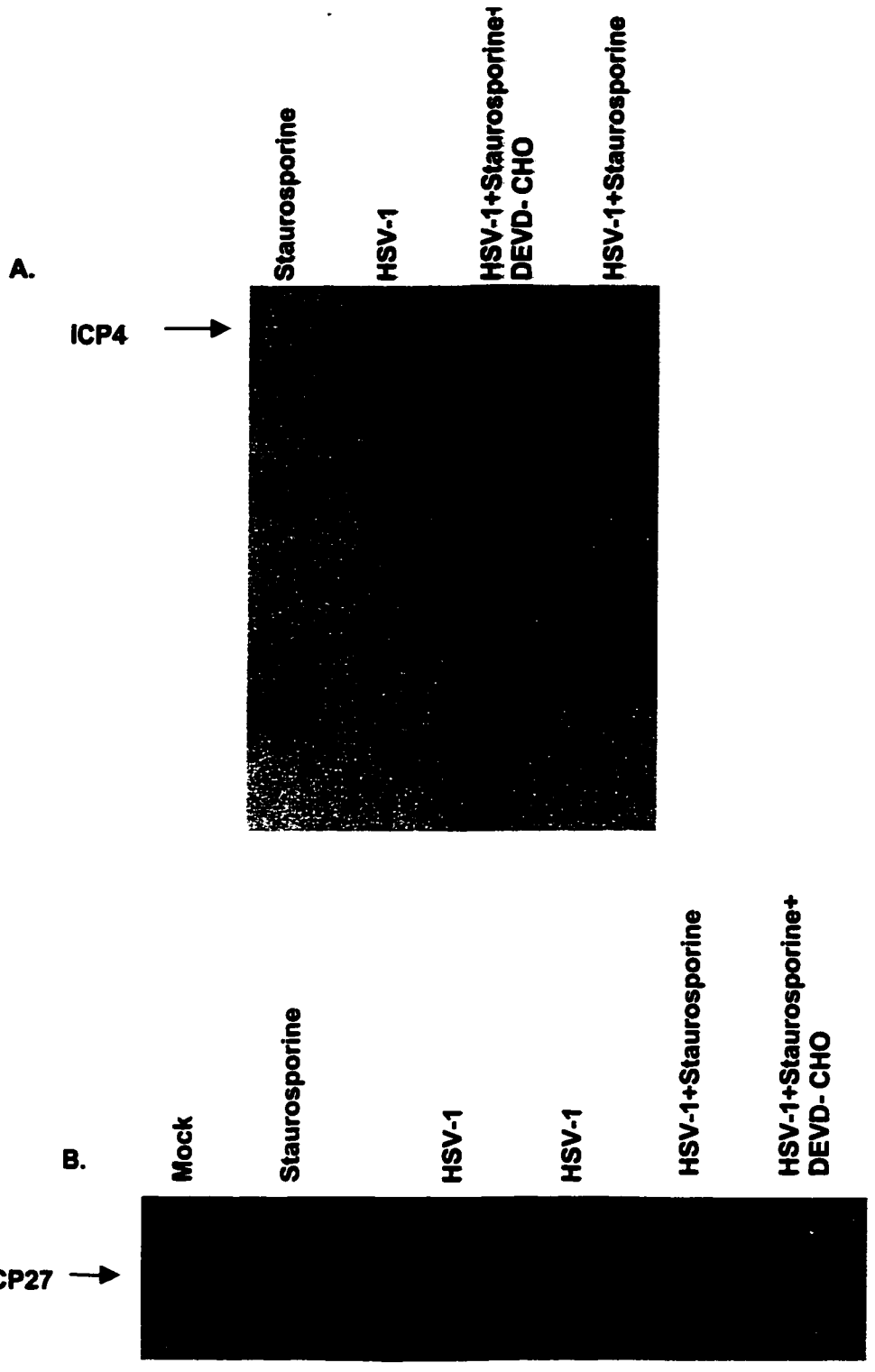

Figure 2.8A and 2.8B Western blot analysis of (A) ICP4 and (B) ICP27 from Vero cell extracts. Vero cells were infected with HSV-1 17+ and subsequently treated with either staurosporine (1uM) with DEVD-CHO (caspase-3 inhibitor 10nM) or staurosporine alone for 6 hours. Arrows correspond to ICP4 band at 175kDa (A) and ICP27 band at 63kDa (B).

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Chapter 3

This research was presented as an oral presentation at the Society for Neuroscience where I was awarded a travel fellowship for this research. This chapter was submitted for publication in Proceedings for the National Academy of Science USA on January 10, 2002.

Capsaicin Induces Reactivation of Latent Herpes Simplex Virus Type 1 in Neurons in Cultures

Abstract

Herpes simplex virus type 1 (HSV-1) produces a life-long latent infection in the neurons of the peripheral nervous system, primarily in the trigeminal and dorsal root ganglia (DRG). Neurons of these ganglia express high levels of the capsaicin receptor, also known as the vanilloid receptor-1 (VR-1). The VR-1 is a sensory neuron, non-selective ion channel that primarily fluxes Ca^{2+} ions in response to various stimuli, including physiologically acidic conditions, heat >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 to the VR-1 channel, capsaicin and heat produced reactivation of latent HSV-1.

Capsaicin-induced reactivation of HSV-1 latently infected neurons was dose-dependent and activation of VR-1 with heat-induced HSV-1 reactivation was temperature-dependent. This heat-induced HSV-1 reactivation was significantly attenuated by treatment with the specific inhibitor to VR-1, capsazepine. These results indicate that HSV-1 reactivation in sensory neurons is induced by VR-1 activation.

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 cause devastating effects upon infection of the eye or the central nervous system. HSV-1 is the leading cause of blindness associated with a viral infection and the leading cause of sporadic fatal encephalitis (6). 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 viral uptake and retrograde transport to the neuronal nucleus occurs. Once HSV-1 infects the peripheral nervous system, it can produce a life-long infection, where the viral DNA remains latent as an episome in the nuclei of the sensory ganglia (1, 24). In humans, diverse stimuli can cause viral reactivation, including fever, prolonged UV exposure or sunburns, severe burns, menstrual cycle and stress (8, 9). Upon reactivation, HSV-1 is anterogradedly transported back to the dermal epithelium to produce lesions and transmissible virus. The mechanism controlling HSV-1 reactivation, to date, remains poorly understood.

In HSV-1 latently infected individuals, reactivation following fever and in burn victims suggests that noxious temperatures may induce a signaling mechanism that leads to reactivation of latent virus. The receptor in the peripheral nervous system that responds to noxious heat stimulus is the capsaicin receptor or VR-1. VR-1 is primarily, but not exclusively, found on nociceptor neurons (3, 13). Nociceptors or pain perceiving neurons exist at the dermal epithelial layer as free nerve endings, in contrast to other peripheral neurons that contain a laminate of some form protecting the nerve ending in the tissue. Because the nociceptor is the only free nerve ending in the dermal epithelium, it may be more susceptible to viral uptake and more likely to harbor latent virus. The exposed free nerve termini of the nociceptor respond primarily to painful stimuli including extreme heat, painful mechanical stimulus and noxious chemicals. These stimuli have been shown to cause HSV-1 reactivation. Nociceptor neurons are not only sensitive to increases in temperatures that exceed 45°C, but are also activated by the pungent extract from hot chili peppers, capsaicin (18).

To elucidate the signaling pathway involved in fever-induced HSV-1 reactivation, we examined the ability of heat shock and capsaicin to induce reactivation 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 (26). Through immunocytochemistry, we found that the majority of the 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.

Material and Methods

Cell culture. Vero cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine serum (Life Technologies, Inc., Rockville, MD). Sensory neuron cultures were prepared from dorsal root ganglia (DRG) of embryonic day 15 Sprague-Dawley rats as previously described (21, 27). Neurons were plated onto 24 well plates at a cell density of approximately $1\text{-}5 \times 10^3$ cells per well. Dulbecco's Modified Eagle's/F12 medium supplemented with 10% newborn bovine serum and 100 ng/ml 2.5 S nerve growth factor (Harlan Bioproducts, Indianapolis, IN) was used to maintain neuronal cultures (neuronal maintenance medium). Cultures were treated with fluorodeoxyuridine (20 μM) for 7-10 days after plating to reduce the non-neuronal cell population.

Establishment of latent HSV-1 infections. Latent HSV-1 infections in neuronal cultures were established as previously described (21, 25, 27). After neurons were established in tissue culture for 2 weeks, 50μM acycloguanosine (Sigma, St. Louis, MO) was added to the culture medium 24 hours prior to inoculation with virus and for the following 7 days after inoculation. Neuronal cultures were infected with approximately 10 plaque-forming units (PFU) of HSV-1 (17^+) per neuron.

Reactivation of latent HSV-1. Reactivation stimuli for latent HSV-1 cultures included capsaicin treatment or heat-shock. Capsaicin (Alexis Biochemicals, San Diego, CA) was

diluted in the neuronal maintenance medium at concentrations ranging from 0.01μM to 10μM and incubated for 4 days prior to harvesting for plaque assays. For heat-shock treatments, latently infected neurons were placed in a 42-46° C water bath for 15 minutes and transferred back to 35°C incubator for 4 days prior to harvesting for plaque assays. For VR-1 inhibition experiments, cultures were pretreated with capsaicin receptor antagonist, capsazepine (Alexis Biochemicals, San Diego, CA), at 10, 25 and 50μM one hour prior to heat-shock at 46°C for 15 minutes. Neuronal cultures were harvested at the times indicated following the treatments indicated and plaque assays were performed. The results presented are from three separate experiments with an n=6 for each treatment group. The data were analyzed using an ANOVA with a Tukey-Kramer adjustment using SAS statistical software.

Cell viability. Cell viability was determined using AlamarBlue (BioSource Int., Camarillo, CA) colorimetric dye according to manufacturer protocol, which changes color based on an oxidation-reduction reaction. The cells were incubated with capsaicin at 1 to 10μM for 4 days and then treated with AlamarBlue for 3 hours. Following AlamarBlue treatment, the medium was collected and read on a colorimetric plate reader at 550nm and 600nm. All treatment groups were compared to 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, St. Louis, MO) and blocked with 5% non-fat milk for one hour. The cells

were then incubated with primary polyclonal rabbit antibody to VR-1 at a concentration of 0.1μg/μl (Oncogene Research, Boston, MA) 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 hour, according to manufacturer's protocol (Vector Laboratories Inc., Burlingame, CA). Diaminobenzidine was used as the substrate.

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% the neuronal cultures were VR-1 positive (Fig.3.1). Additionally, VR-1 staining showed that the VR-1 receptor was not only located on the cell body of the neurons, but also on the neurites and the nerve terminals.

Capsaicin Treatment Induced Reactivation of Latent HSV-1

To determine the effects of VR-1 on the latent HSV-1 infection, the neurons were treated with capsaicin, the potent agonist of the VR-1 channel. In the *in vitro* neuronal model of HSV-1 latency, capsaicin treatment caused reactivation in a dose-dependent manner, using concentrations of 0.01μM to 0.1μM (Fig.3.2). However, at high concentrations of capsaicin (10μM and 1μM) there was a significant decrease in reactivation following treatment. Capsaicin treatment produced a bell shaped dose-response curve for HSV-1

reactivation. The AlamarBlue cytotoxicity assay indicated that no significant cell death was observed following capsaicin treatment at concentrations of 0.1μM, 1μM and 10μM over the four-day treatment period (Fig.3.3).

Heat Shock- Induced Reactivation of Latent HSV-1 is Temperature Dependent

VR-1 activation normally occurs at temperatures exceeding 45°C. To determine the effects of heat shock on neuronal cultures harboring latent HSV-1, the cultures were subjected to heat stimulation between 42-46°C (Fig.3.4). Following heat stimulation at 46°C, we observed a 5000-fold increase in viral titers. Reactivation was not induced at temperatures below 42°C. Temperatures of 44°C and 45°C produced approximately 40-fold increase in viral titers. Reactivation of latent HSV-1 was temperature-dependent, with the greatest viral titers produced by heat shock 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 specifically determine the role of VR-1 in heat shock induced HSV-1 reactivation, the specific VR-1 antagonist capsazepine was used to block heat shock-induced reactivation. Neuronal cultures harboring latent HSV-1 were treated for 1 hour with capsazepine prior to heat shock at 46°C. Following heat shock, the neurons were assayed for infectious virus (Fig.3.5). The data indicated that treatment with capsazepine significantly reduced reactivation of latent virus in a dose-dependent manner. These data further suggest that reactivation following heat treatment involved the activation of the VR-1 receptor.

Discussion

HSV-1 reactivation has been studied in many *in vivo* and *in vitro* models. The mechanism underlying the critical pathways involved in the switch between latent virus and reactivated virus is not yet understood. Studies presented here indicated that calcium-dependent signaling pathways might play a key role in these mechanisms. VR-1 is an ion channel that preferentially fluxes calcium in response to capsaicin treatment. This receptor is found on nociceptor neurons, which are part of the peripheral sensory nervous system. Upon activation, VR-1 can cause depolarization of the sensory neuron generating an action potential in response to increased Ca^{2+}. The neuronal cultures derived from the DRG are a mixed neuronal population that contains nociceptor neurons as well as other sensory neurons, although our results indicate that the vast majority of neurons in culture are VR-1 positive. Nociceptor neurons are important for the sensation of different pain stimuli including pressure, heat exceeding 45°C, pH changes and noxious compounds, such as capsaicin.

In humans, fever is a common stimulus for HSV-1 reactivation (8). Furthermore, in animal models, heat shock produces reactivation of latent HSV-1 (14, 17). However, the mechanism of heat-induced reactivation of HSV-1 has not been elucidated. 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.

Capsaicin has been used as a treatment to alleviate the recurrence of herpetic lesions in a guinea pig model of HSV latency (2, 22). In these experiments, HSV-2 was inoculated vaginally in guinea pigs and the severity of the herpetic lesions and recurrence was assessed using a visual scoring method. 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 (22). 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, although our studies show that capsaicin induced HSV-1 reactivation, we also noted a desensitization of VR-1 at higher concentrations of capsaicin. Furthermore, our data suggest that VR-1 may play an important role in heat-induced reactivation of latent HSV-1, since the specific inhibitor of the capsaicin receptor, capsazepine, decreased viral reactivation in a dose-dependent manner. These data imply the importance of VR-1 and nociceptors in the induction of reactivation of latent HSV-1.

In our studies, we have found that capsaicin induced HSV-1 reactivation in a bell shaped dose-response curve. Since there was minimal toxicity detected at the higher concentrations of capsaicin, we attributed this altered reactivation kinetics to desensitization of the VR-1 channel following high-dose treatment. These data may

suggest a protective mechanism of the neuron to shut off damaging Ca^{2+} influxes. Other investigators have noted desensitization of VR-1 following repeated stimulation in electrophysiology experiments (7, 11, 15). Furthermore, other studies noted a decrease in VR-1 mRNA levels following capsaicin treatment, suggesting that down regulation of the receptor occurs (13). The mechanism underlying this desensitization has not been elucidated, although it has been suggested that capsaicin desensitization may be similar to calcium-dependent inactivation of the N-methyl-D-aspartate receptor (7). Because VR-1 displays rapid desensitization in electrophysiology experiments and long-term desensitization in animal models, there may be two components of capsaicin desensitization. One component may be attributed to receptor binding regions of the ligand that change the kinetics and gating properties. The second component may involve actual regulation and receptor turn-over properties. These two components may regulate the internal calcium levels to ensure survival of the neuron.

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 to a common event. Previous results demonstrated the roles of protein kinase C (PKC) and protein kinase A (PKA) in reactivation of latent HSV-1 (20, 27). Here we demonstrated that treatment of latently infected neurons with capsaicin or heat shock also induced reactivation of HSV-1 latently infected neurons in culture. A common feature of these stimuli is hyperexcitation of the neuron. For example, forskolin induces up-regulation of PKA, which is known to activate the VR-1 receptor (12). Phorbol 12-myristate 13-acetate (PMA) activates the second messenger PKC, which

can directly stimulate sensory specific sodium channels and cause excitation of the neuron (5, 10). PMA compounds are also known to activate VR-1 (16, 23). Heat and capsaicin stimulation directly activates VR-1, which can cause depolarization and firing of the neuron (4). All of these events include some aspect of excitation of the neuron. Finally, the *in vitro* neuronal latency model was initially characterized using a stress stimulus of nerve growth factor (NGF) deprivation to induce HSV-1 reactivation of latently infected neurons (12). NGF deprivation may involve activation of the low affinity NGF receptor, p75, which is also reported to modulate VR-1 activity (19).

In conclusion, noxious stimulation of nociceptors via heat or capsaicin may cause increases in calcium concentrations in the neuron that are critical for the induction of HSV-1 reactivation. In our studies, VR-1 activation through both capsaicin and heat led to HSV-1 reactivation that could be blocked using an antagonist to VR-1, capsazepine. These data indicate that VR-1 plays an important role in HSV-1 reactivation.

Acknowledgements:

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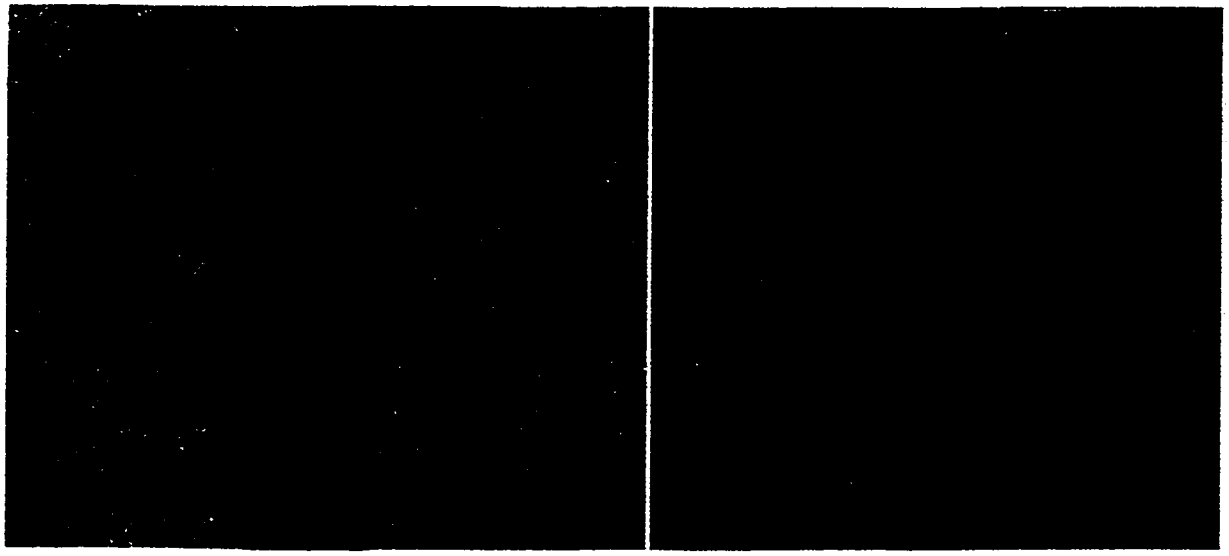

Figure 3.1. Immunocytochemistry using anti-VR-1 polyclonal antibody demonstrated the abundance of VR-1 positive neurons in the DRG culture (A). In addition to the cell bodies staining positive for VR-1, the neurites and the nerve terminals (arrows) were also VR-1 positive (B).

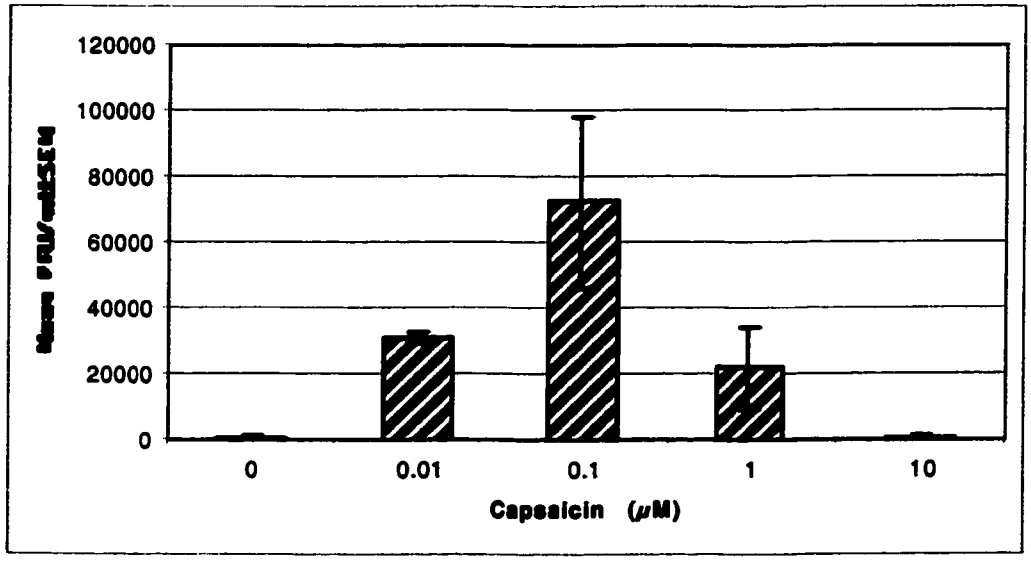

*Figure 3.2 Capsaicin induced reactivation of HSV-1 latently infected neuronal cultures. The data indicates that at 0.1μM there was a 70-fold increase from no treatment control and the difference was statistically significant based on an ANOVA with a Tukey Kramer adjustment * (p=<.0001). The data are representative of three separate experiments with an n=6.*

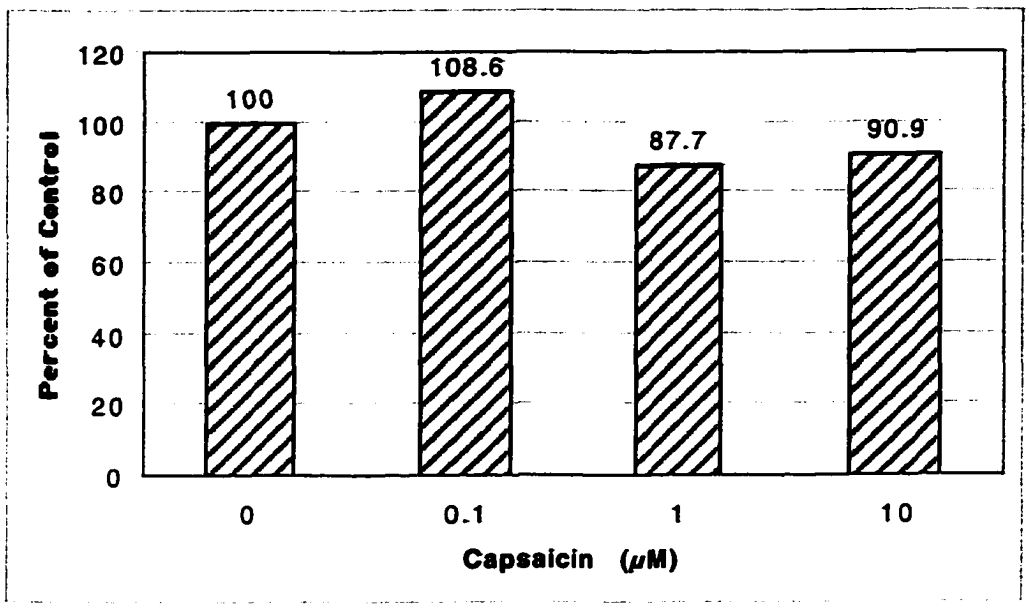

Figure 3.3 Capsaicin did not cause significant cell death, which was measured using AlamarBlue viability dye. Each treatment group consisted of n=6 and the data showed no statistically significant cell death in any of the treatment concentrations tested (0.1, 1, 10μM).

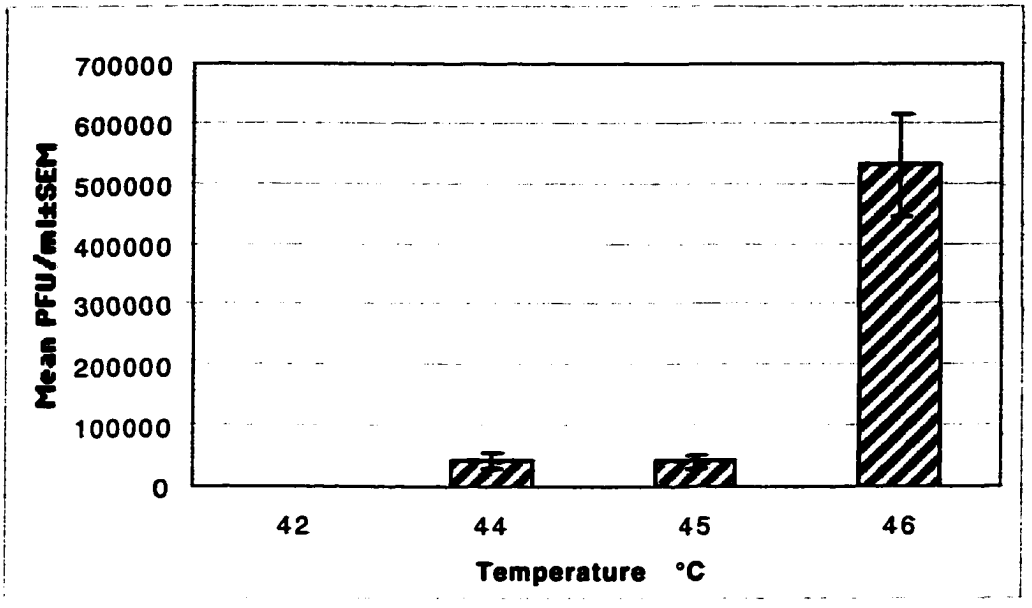

*Figure 3.4 Heat shock induced reactivation of HSV-1 latently infected neuronal cultures. The data indicates a 5000-fold increase from 42°C to 46°C, which was statistically significant based on an ANOVA * (p=<.0001). Each group represents n=6.*

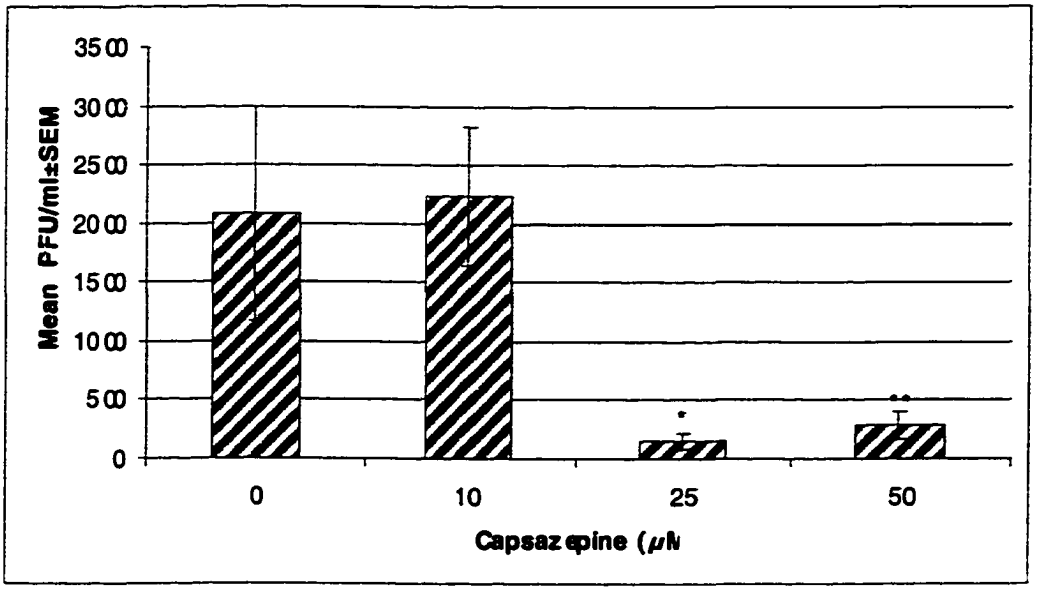

*Figure 3.5. Heat shock induced reactivation of HSV-1 latently infected DRG culture that were pre-treated with VR-1 specific inhibitor, capsazepine. The data indicate that a block in heat-induced reactivation occurred at capsazepine concentrations of 25 and 50μM. Statistical significance using a one-way ANOVA between no treatment control and 25μM *(p=.025) and 50μM **(p=.045) was observed.*

Appendix to Chapter 3

The appendix discusses studies that were not included in the manuscript for various reasons including insufficient or negative data but has important relevance to the overall significance of the work in Chapter 3.

The effects of intracellular calcium stores on HSV-1 reactivation

Introduction

In order to address the role of intracellular calcium stores in HSV-1 reactivation, I used two different pharmacological agents that induce calcium release from intracellular stores. These two pharmacological agents included cyclopiazonic acid (CPA) and thapsigargin. Both compounds are known to release calcium by inhibiting the calcium ion pump proteins of intracellular membranes located in the ER. Thapsigargin is a plant derived compound from the plant Thapsia garganica L and it is normally fast acting changing intracellular calcium levels in the neuron fairly rapidly compared to the leaky nature of calcium release following CPA treatment. If intracellular calcium levels are critical for HSV-1 reactivation then increasing calcium by poisoning the ER calcium ion pumps should in theory cause HSV-1 reactivation.

Materials and Methods

DRG neurons were latently infected with HSV-1 as previously described. HSV-1 reactivation was induced with treatment of thapsigargin at concentrations of 5, 2.5, 1μM and CPA, tested at concentrations of 200, 100, 50, 25μM. Five days following treatment the neurons were assayed for infectious viral particles using a standard plaque assay.

Results and Discussion

Our results indicated that both thapsigargin and CPA had no effect on latent HSV-1 and did not induce reactivation. We attributed these results to the fact that DRG neurons do not store large quantities of calcium in either the mitochondria or the ER. Also, CPA causes a very slow release of calcium even in muscle cells, which have large stores of calcium (personal communication by Jennifer O'Brien). Therefore, increases in intracellular calcium levels by these compounds were probably not significant enough to cause an effect. Moreover, the neuron was probably able to use plasma membrane ion channels to decrease toxic intracellular calcium levels induced by the ER poisons.

Experiments by Shmigol et al., 1995 studied the effects of thapsigargin on calcium currents and intracellular stores in DRG primary cultures (2). They determined that thapsigargin did not cause any substantial changes in basal intracellular calcium levels but did irreversibly inhibit caffeine mediated calcium release from intracellular pools. Surprisingly, the authors conclude that thapsigargin has a dual action affecting intracellular calcium pools and inhibiting extracellular calcium entry by deactivating high-voltage-activated Ca^{2+} channels. Since we were unable to actually measure

intracellular Ca^{2+} in the neuron, we speculate that thapsigargin and CPA did not have an impact on intracellular Ca^{2+} levels and therefore did not cause HSV-1 reactivation. Future studies should include fluorescent measurement of intracellular Ca^{2+} to verify our assumptions.

BAPTA attenuates forskolin-induced HSV-1 reactivation

Introduction

Recent studies determined that administration of BAPTA together with forskolin treatment reduced viral titers significantly in the *in vitro* latency model. BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid Tetra(acetoxymethyl) Ester) is a membrane permeable form of BAPTA that is hydrolyzed by cytosolic esterases and trapped intracellularly as the active calcium chelator. We had hypothesized that forkolin may be regulating calcium ion channels via phosphorylation and therefore its effects on HSV-1 reactivation in addition to elevating cAMP levels is regulation of intracellular calcium through plasma membrane ion channels. To determine the validity of this hypothesis we tested the effects of the calcium chelator BAPTA following forskolin-induced HSV-1 reactivation.

Materials and Methods

The same procedure was followed as those presented in Chapter 3 for cell culture and establishment of latency. The neurons were maintained in latency for the designated 14 days and reactivated with 100μM forskolin in the presence of 100μM BAPTA. A standard plaque assay was used to determine infectious viral particles present following the reactivation stimuli.

Results and Discussion

BAPTA-AM is a cell permeable calcium chelator and an efficient calcium chelator compound compared to EGTA. In the presence of BAPTA-AM, forskolin-induced reactivation was reduced over 2-fold compared to forskolin alone (Fig.3.6). This datum implies that the mechanism of HSV-1 reactivation induced by forskolin involves intracellular calcium levels and that forkolin through some unknown mechanism initiates calcium influxes into the cell that may be excitatory. Additionally, this datum suggests that important proteins necessary for HSV-1 reactivation not only require phosphorylation by PKA but also require calcium.

Capsazepine attenuates forskolin-induced HSV-1 reactivation

Introduction

We designed experiments to test the hypothesis that forskolin regulated the VR-1 channel through phosphorylation. In order to test this hypothesis we treated the DRG cultures with the VR-1 antagonist capsazepine prior to forskolin treatment.

Materials and Methods

The same procedure was followed as those presented in Chapter 3 for cell culture and establishment of latency. The neurons were maintained in latency for the designated 14 days and reactivated with 100μM forskolin in the presence of 50μM capsazepine. A standard plaque assay was used to determine infectious viral particles present following the reactivation stimuli.

Results and Discussion

The VR-1 antagonist, capsazepine attenuated and in some cases completely abolished reactivation induced by forskolin. This datum strongly suggests that VR-1 gating properties and channel activation involve phosphorylation via PKA. Therefore, forkolin not only increases cAMP levels that may be essential for important regulatory proteins involved with the initiation of transcription of immediate early genes but forkolin also regulates calcium influxes in the cell that are even more critical for HSV-1 reactivation. This datum further supports the effects of forskolin as an excitatory stimulus that is calcium dependent and mediated via VR-1 resulting in reactivation of latent HSV-1 (Fig.3.6).

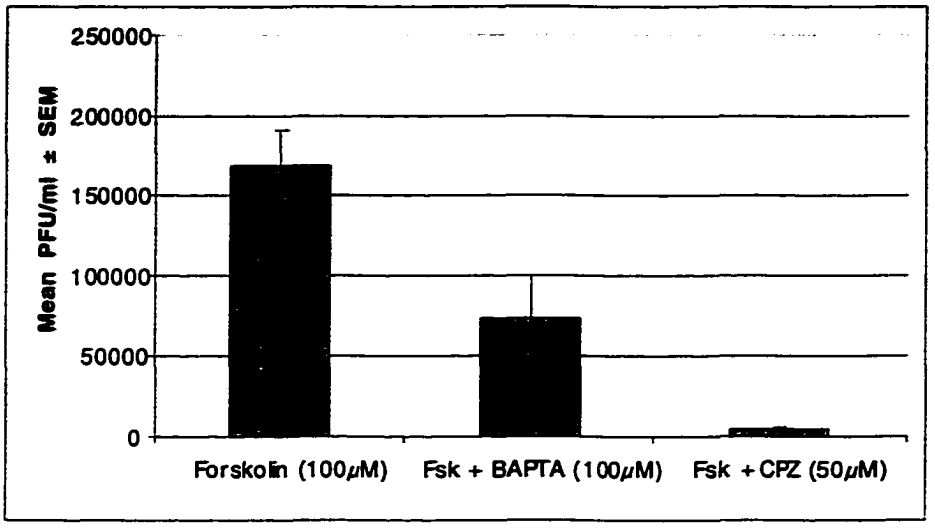

Figure 3.6 BAPTA and capsazepine inhibit forskolin-induced HSV-1 reactivation from latently infected DRG cultures.

Capsaicin-Induced Synaptic Plasticity

Introduction

Capsaicin induced a bell-shaped dose-response curve that we attributed to desensitization of the VR-1 channel at the higher concentrations of capsaicin (1 and 10μM). This desensitization may be due to channel down regulation at the higher capsaicin concentrations.

Materials and Methods

In order to address this theory of channel desensitization due to down regulation of VR-1, we analyzed the protein levels of VR-1 in the DRG cultures 5 days after treatment with capsaicin. We followed the same protocol as the latency model except the neurons were not latently infected. Following capsaicin treatment at 0.1, 1 and 10μM, the neurons were harvested for protein extracts. The DRG cell extract was run on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. A monoclonal antibody to rat VR-1 (Santa

Cruz, SC-8671) was used for immunoblotting at the manufacturer's suggested concentration of 1:200.

Results and Discussion

Immunoblotting for VR-1 showed an increase in VR-1 expression at lower concentrations of capsaicin (1 and 0.1μM) (Fig. 3.7). Densitometry of the western blot provided quantitative results of these increases outlined in Table 3.1. Although we should be careful in our analysis of these kinds of results, we are tempted to assume that the decrease in protein levels at the higher concentrations of capsaicin imply down-regulation of VR-1 expression. Furthermore, although we assume that the antibody is specifically binding to VR-1, the stained in our immunoblot was larger than the predicted size based on the amino acid sequence. This result may suggest that the protein underwent post-translational modification (1).

VR-1 as an HSV-1 entry molecule

Experiments examining VR-1 as an entry molecule were inconclusive. A CHO-K1 cell line was transfected with VR-1 over-expressing plasmid and subsequently infected with a recombinant HSV-1 virus that expressed ICP4-GFP. This hamster cell line was previously characterized as an HSV-1 non-permissive cell line. Unfortunately, in my experiments I determined that indeed our recombinant viruses could infect these cells without the presence of VR-1 hence, these cells were not HSV-1 non-permissive.

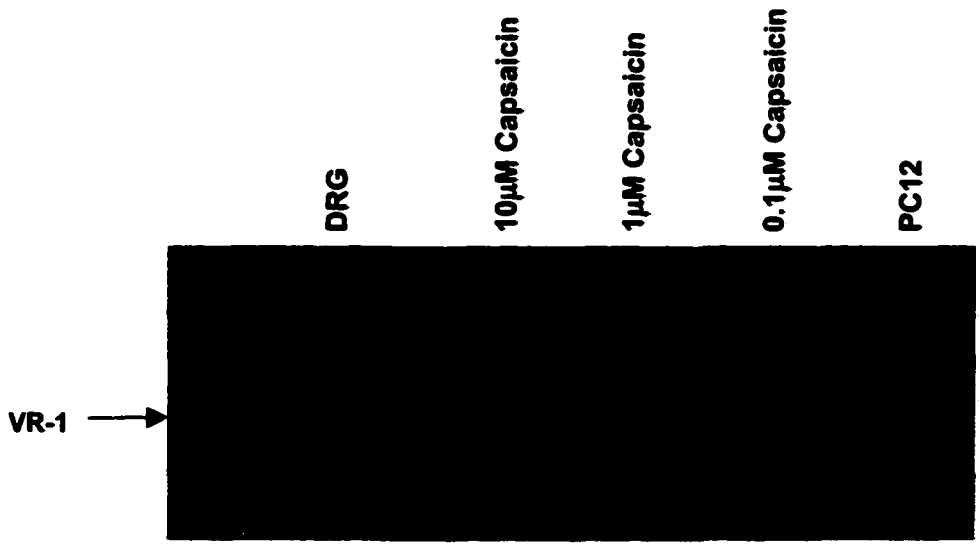

Figure 3.7 Western blot analysis of VR-1 expression in DRG cultures treated with capsaicin for 5 days. PC12 cells did not express the VR-1 receptor.

Treatment Group	Pixel/0.01Sq inch
DRG	15.8
DRG+ Capsaicin 0.1μM	20.68
DRG+ Capsaicin 1μM	18.33
DRG+Capsaicin 10μM	13.44

Table 3.1 Densitometry analysis of western blot analysis of capsaicin induced synaptic plasticity.

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Chapter 4

Cyclin D1 Expression in DRG Neurons

Abstract

Herpes simplex virus type 1(HSV-1) has been implicated in altering the cell cycle following a primary infection. Alteration of the cell cycle involves synthesis of cyclins and cyclin depedent kinases, such as cyclin D1, cyclin D3 and cdk2. These proteins are known to drive the cell from G1 to S phase. Although the role of these proteins may be important in a lytic infection, their role in reactivation following a latent infection has not been elucidated. HSV-1 targets peripheral neurons, a terminally differentiated cell, and becomes latent within this cell type. Initiating replication in a neuron through activation of the cyclin proteins can result in apoptosis. Immunocytochemistry experiments indicated that cylin-D1 expression in rat DRG neurons is very low and only detectable using signal amplification systems. Cyclin-D1 expression was diffuse and not compartmentally localized to the nucleus as expected. Moreover, cyclin-D1 was not induced following an apoptotic signal of NGF deprivation in the DRG neurons.

Introduction

HSV-1 belongs to the alphaherpesviridae family of viruses that are notorious for latent infections in the sensory nervous system. HSV-1 tropism is the PNS, although retrograde transport to the CNS can occur. A latent infection only transpires in the PNS whereas a CNS infection results in devastating life-threatening effects such as encephalitis. The difference that enables HSV-1 to establish a latent infection in the PNS and not the CNS is not known. Cells from both the CNS and PNS are terminally differentiated, but there is some evidence that CNS neurons are capable of regenerating in some manner (reviewed by (6)). The differences between these two types of neurons lie in their capability to divide following maturation and throughout the life of the organism.

Replication of a normally dividing cell depends on the presence of cyclin proteins in the nucleus. In a favorable replication environment, the dividing cell transitions from a G0 arrest state into G1 and S phase where DNA replication takes place. The key element that transitions the cell from G1 to S phase is the expression of cyclin-D1 and cyclin dependent kinase (cdk) 4/6. Together these two proteins form a holoenzyme complex that phosphorylates retinoblastoma protein (Rb) and releases E2F to begin DNA synthesis thus, facilitating the progression from G1 to S-phase (7). Cyclin-D1 has a very short half-life and is continuously phosphorylated on Thr-268 by glycogen synthetase kinase 3B (GSK-3B). Phosphorylation of cyclin-D1 exposes polyubiquitination sites, targeting the protein for degradation. The destruction of cyclin-D1 halts the progression from G1 to S-phase hence the cell remains in G1 arrest (2, 3).

In 1994, Freeman et al. developed the first hypothesis that sensory neurons underwent apoptosis in response to a replication stimulus (5). Because of the

unavailability of specific cell cycle protein antibodies, these studies mainly relied on RT-PCR, in situ hybridization and northern blot analysis to determine the regulation of cell cycle proteins in sensory neurons. Their data indicated that in response to nerve growth factor (NGF) withdrawal, the neurons underwent apoptosis that correlated to an increase in cyclin-D1 RNA. There were no further studies to confirm this hypothesis and the role of cyclin proteins in neurons has yet to be determined.

Our preliminary immunocytochemistry studies confirm the presence of cyclin-D1 in the mature DRG neuron. Interestingly, studies using confocal microscopy could not detect cyclin-D1 in the nucleus of the neuron. Contrary to Freeman and colleagues, we could not induce a detectable increase in cyclin-D1 protein expression following NGF withdrawal.

Materials and Methods

Cell culture

Same as Chapter 2 and 3.

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, St. Louis, MO) and blocked with 5% non-fat milk for one hour. The cells were then incubated with primary monoclonal rat antibody to cyclin D1 at a concentration of 0.1μg/ml (Pharmingen) for 2 hours at room temperature. Following primary antibody incubation, the cells were washed and incubated with a universal secondary IgG anti-rabbit antibody conjugated to horseradish peroxidase for 1 hour

according to manufacturer's protocol (Vector Laboratories Inc., Burlingame, CA). Alkaline phosphatase and DAB were used as substrates.

Confocal Microscopy

DRG neurons were matured in culture for 14 days and then deprived of NGF for various time periods (1, 2, 6, 12 and 24 hours). Cultures were fixed and stained using primary antibodies against cyclin-D1 (Pharmingen) and with a secondary antibody IgG anti-mouse with a FITC fluorescent tag (Molecular Probes). Cultures were examined for the presence of cyclin-D1. There was no detectable level of cyclin-D1 observed in these cultures.

Western Blot Analysis of Cyclin-D1

Neuronal cultures were deprived of NGF for 1,3 and 24 hours. The cell extracts were harvested using RIPA buffer with protease inhibitors. The proteins were separated by electrophoresis on a polyacrylamide gradient gel containing a positive control of primary rat fibroblasts that were induced to cycle simultaneously. Briefly, the rat fibroblasts were grown to sub-confluency and placed in 0.5% fetal bovine serum (FBS) in DMEM (Gibco BRL, Rockville, MD) for 48 hours. Following serum deprivation, the cells were shocked with 10% FBS-DMEM for 24 hours to induce synchronous cell division and harvested for protein extract. Following electrophoresis, the protein was transferred to a nitrocellulose membrane for immunoblotting. The membrane was immunoblotted with monoclonal primary antibodies to cyclin-D1 (BD-Pharmingen, San Diego, CA) at the manufacturer's suggested concentration of 0.1μg/ml. The secondary antibody was an

anti-mouse IgG conjugated to horseradish peroxidase. Standard chemiluminesence detection method was used to visualize cyclin-D1 bands on the gel (NEN Life Science Products, Inc., Boston, MA).

Results

Immunocytochemistry and confocal microscopy indicate no differences in cyclin-D1 expression in DRG cultures treated with anti-NGF and control.

Based on immunocytochemistry, the pilot studies indicated that approximately 30% of the neurons expressed cyclin-D1. This expression was not confined to the nucleus as previously reported by other investigators (Fig 4.1). Four different experiments indicated that NGF deprivation did not alter cyclin-D1 expression in the neurons. One experiment suggested that latent neurons had no expression of cyclin-D1 (Table 4.1). Further analysis using confocal microscopy indicated no staining of cyclin-D1 in both control and anti-NGF samples.

Western blot analysis showed no detectable levels of cyclin-D1 in DRG cultures.

Rat primary fibroblasts were serum deprived for 48 hours and then serum shocked for 24 hours in order to induce detectable levels of cyclin-D1 by western blot analysis. Samples included neuronal cultures that were either latent or reactivated with NGF withdrawal. We could not detect any cyclin-D1 in these samples by western blot analysis (Fig. 4.2).

Discussion

The mechanism of HSV-1 reactivation in neurons has not been elucidated to date. Some hypothesize that the level of immediate early protein expression is altered in the neuron, hence the viral DNA becomes latent and remains in the nucleus as an episome. Others postulate that there are differences in Oct-1 expression or repressors of Oct-1 in neurons that mediate latency. But the difference between a productive infection and latent infection may entail the differences between a terminally differentiated cell, such as the neuron and an actively dividing cell such as an epithelial cell. An actively dividing cell does not have the same protein expression as a terminally differentiated cell. There may be differences in gene expression requiring different transcription factor expression. Investigating the effects of cycling proteins may unveil the subtle differences that are necessary for the establishment of a latent infection.

The most recent literature supports two separate hypotheses of the role of cyclin proteins during a lytic infection. Roizman et al., (2000) hypothesize that cyclin D3 stabilizes ICP0 at a specific aspartic acid residue, which allows continual immediate early gene transcription. In addition, Van Sant et al., (2001) also hypothesize that cyclin D3 can replace ICP0 for viral replication, particularly if a cell is in transition from G1 to S-phase (8, 9). Similarly, Ehman et al., (2001) hypothesize that HSV-1 stops the normal cell cycle specifically from proceeding from G1 to S phase (4). Their research also speculates that VP16 or alpha TIF requires cdk2 in order to be functional. These data support the idea that the cyclin proteins along with the cdk proteins play a role in productive infection in an actively dividing cell. But what is their function in a non-dividing or terminally differentiated cell such as a neuron?

Freeman et al., 1994 first described the expression of the cyclin proteins in the neuron (5). Their data indicated that cyclin D1 expression increased in response to NGF withdrawal whereas cyclin D3, D2 C, B and cdk 5 and 4 all decreased. The authors hypothesized that in order for neurons to remain terminally differentiated the neurons response to a cell division signal is apoptosis. Hence, the neuron initiates programmed cell death in response to a cell division signal. Using this model we proposed that the NGF signaling pathway play an important role in HSV-1 reactivation in neurons. We assumed that during NGF deprivation the up-regulation of the cyclin proteins may transition the virus from latency to reactivation. The signaling pathways that are disrupted following NGF withdrawal are mediated via the TrkA receptor.

The DRG neuron has two important receptors that recognize and signal upon NGF binding. The P140 receptor, also known as TrkA, is important for cell survival and maintenance. Blocking this receptor induces cell death. The signaling pathways proposed for this receptor occur either through the MAPK pathway or through PI-3K pathway. The MAPK pathway involves activation of the adaptor protein, Ras. Ras signaling is known to regulate cyclin-D1 in various ways. Ras induction can turn on the PI-3K pathway and cause up-regulation of Akt and subsequent phosphorylation of cyclin-D1. This phosphorylation causes cyclin-D1 to be exported out of the nucleus where it is ubiquitinated and degraded by proteosomes. Conversely, Ras up-regulation can cause increased cyclin-D1 expression followed by an increase in D-type kinases. This increase in cyclin-D1 phosphorylates the retinoblastoma protein (Rb), releasing the transcription factor E2F, essential for DNA replication (1). In order to maintain basal nuclear levels of cyclin-D1, the cell is simultaneously synthesizing and degrading cyclin-D1.

Data from our laboratory using recombinant Ras adenoviruses indicated that overexpression of constitutively active Ras protein caused HSV-1 reactivation in DRG latently infected cultures (data not shown). These data implies that NGF signaling pathway in DRG neurons does not rely on Ras activity. Studies with cyclin-D1 did not show fluctuations of protein expression during NGF withdrawal. In fact, western blot analysis indicated that cyclin-D1 cannot be detected in the neuron following NGF deprivation and immunocytochemistry experiments showed no changes in cyclin-D1 expression during NGF deprivation. Furthermore, confocal microscopy analysis showed no detectable nuclear cyclin-D1 in the neuron, which may be due to the low abundance of the protein that could not be visualized without amplification.

In conclusion, NGF is an important neurotrophic factor that is essential for the maintenance and survival of neurons both in culture and *in vivo*. The signaling pathway following NGF deprivation does not appear to include cyclin protein regulation. Although previous literature indicates that cyclin-D1 can substitute for ICP0, this is probably not the case in neurons. Therefore, we conclude that cyclin-D1 has no role in HSV-1 reactivation in neurons and its role in herpes replication must be restricted to actively dividing cells during a lytic infection.

Figure 4.1 Immunocytochemistry detecting cyclin-D1 in DRG cultures. The staining demonstrates both nuclear and cytoplasmic localization of cyclin-D1(A). Control DRG cultures with no primary antibody treatment and only secondary antibody treatment demonstrates no non-specific staining (B).

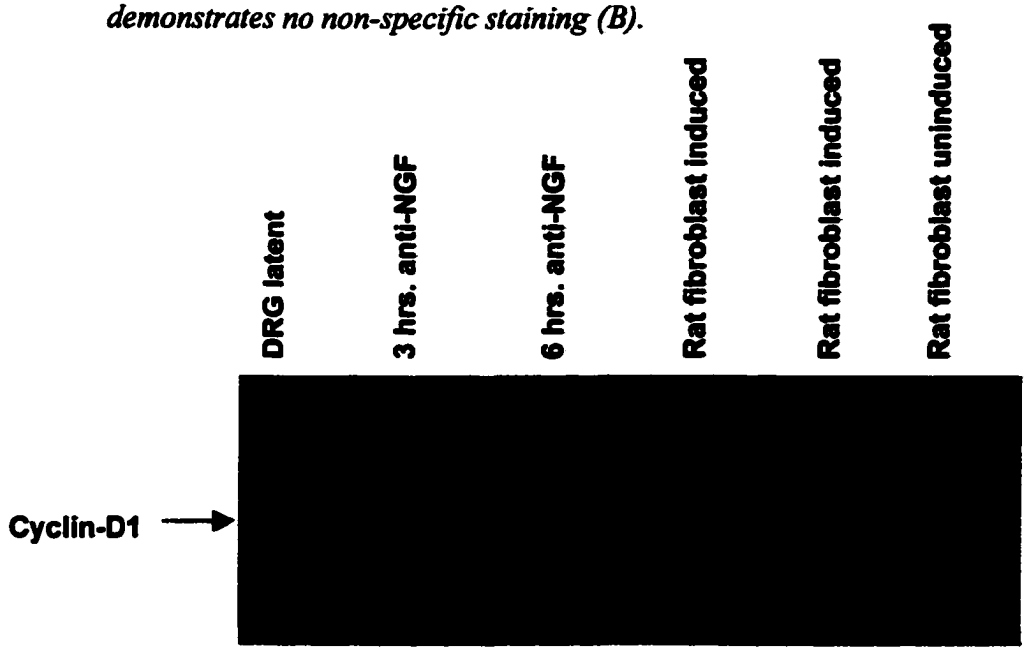

Figure 4.2 Western blot analysis for cyclin D1. HSV-1 latently infected DRG cultures were treated with anti-NGF for 3 and 6 hours to induce cyclin-D1 expression. Control cultures included rat primary fibroblast induced by synchronizing cell division and uninduced. The gel indicates a doublet band at 36kDa that is normally observed in rat tissue. No detectable band was observed for all DRG samples tested.

Treatment Groups	% Positive D1 Staining ±SD
Latent	8.9±11.5
6 hours Anti-NGF	16.2±22.7
24 hours Anti-NGF	11.5±5.5

Table 4.1 Immunocytochemistry quantitative analysis of cyclin D1 positive DRG neurons. Seven random fields were analyzed for cyclin D1 positive neurons and represented as a percentage of total cells in the field

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Chapter 5

Conclusion and Future Directions

Does neuronal cell biology contribute to HSV-1 latency

HSV-1 remains latent predominantly in sensory neurons of the peripheral nervous system. So, what makes a neuron different than a normally dividing cell? The difference involves two physiological aspects that set neurons apart from all other cell types. First, neurons are terminally differentiated cells and have silenced the genes necessary for DNA synthesis. Because the neuron does not divide, its DNA is not arranged in compact structures but instead exists uncoiled and loosely arranged throughout the nucleus. This uncoiled DNA may account for the fact that neurons transcribe 10-20 times more mRNA sequences than kidney or liver cells. Second, they conduct electrical signaling over a long distance. This electrical signaling involves specific ion channels and intracellular ion regulation for membrane potential changes. Furthermore, the neuron synthesizes neurotransmitter proteins that serve as a chemical signal between synapses. In the peripheral nervous system, the cell body of a neuron can be several feet away from the end terminal at the periphery. So the neuron has complicated cytoskeletal architecture compared to other cells. Additionally, the neuron can translate mRNA at either the

dendrite or the terminal for de novo protein synthesis. But are these differences important for the establishment of a latent infection?

It has been suggested that HSV-1 remains latent in sensory neurons because the virus requires the cell cycle for efficient replication. But, HSV-1 is cell cycle independent. Also, HSV-1 uses all of its own replication machinery (3). In fact, recent studies by Ehman et al., 2000 demonstrated that HSV-1 halts the cell cycle during a productive infection (7). Perhaps latency is due to the long journey the virus must ensue from the periphery to the neuronal cell body. These long axons contain different microtubule motors necessary for axonal transport of essential proteins and organelles, such as the mitochondria. Therefore, upon primary neuronal infection, the essential proteins necessary to initiate transcription of viral DNA, such as VP16 never arrive at the nucleus prior to cellular degradation. Also, these proteins may not contain the essential microtubule associated sequences that allow expedient exportation to the nucleus along with the capsid. Hence, the essential transactivator VP16, along with the other immediate early proteins, are not present when the viral DNA reaches the nucleus. This would fit nicely with the latency hypothesis of insufficient IE. At some point neuronal factors may actually silence viral DNA and maintain it in dormancy. These silencing factors may involve histone association, methylation of herpes DNA or other neuronal specific proteins involved in gene silencing.

Is HSV-1 latency established in a particular neuronal sub-type? Studies in mice identified latently infected neurons containing SSEA-3+ surface marker more likely to harbor latent virus over CGRP+, Substance P+ and LD2+ (10). The authors did not stain for VR-1 positive neurons but did identify TrkA positive neurons, which are often pain

neurons. We hypothesize that nociceptor neurons are more susceptible to HSV-1 initial infection because they exist as free nerve endings in the dermal epithelium. Second, the stimuli that cause herpes reactivation highly correlate with nociceptor stimulation. For example skin burns, sunburns and skin abrasions all excite the nociceptor neuron and cause herpes reactivation in humans. Furthermore, in humans, primary infection is associated with hyperalgesia. Although studies discussed here did not demonstrate conclusively that nociceptors are more susceptible to herpes infection, circumstantial evidence indicated that HSV-1 reactivation stimuli also excite nociceptor neurons. To confirm that VR-1 positive neurons are more likely to harbor latent HSV-1, future studies would entail correlating LAT positive neurons with VR-1 positive cells either *in vivo* or *in vitro*. The mouse ocular model could be used for these experiments since these animals establish latency and reactivate. In situ hybridization for HSV-1 DNA in combination with VR-1 immunocytochemistry should co-localize HSV-1 positive latent neurons with VR-1 positive neurons and provide further evidence to support the nociceptor hypothesis.

The Unifying Calcium Hypothesis

Since the presence of the VR-1 calcium channel determines whether a neuron is a nociceptor, calcium regulation may be the key element necessary for herpes reactivation. In our model, intracellular calcium levels appear to be a unifying event inducing the reactivation of HSV-1 from a latent infection. Intracellular calcium levels modulate herpes reactivation via either a direct role through calcium dependent transcription factors or through an indirect activation of host cell gene transcription that serendipitously initiates viral replication. Based on our findings, these calcium levels

must be sufficient to cause neuronal modulations or changes in neuronal plasticity in order to cause reactivation of latent HSV-1.

Calcium is highly regulated in neurons because of its pleitropic effects. Calcium regulation is critical for signal transduction, neurotransmitter release, excitatory potentials, differentiation, migration, synaptic plasticity and general survival of the neuron. Therefore, regulation of calcium is of utmost importance and its regulation differs from other cell types. The neuron does not store large amounts of calcium in its organelles like the muscle cells do in the sarcoplasmic reticulum. In the neuron, the mitochondria store relatively small amounts of calcium and during a calcium overload the neuron is unable to sequester damaging levels of calcium. Therefore, the neuron relies on calcium channels and non-selective ion channels such as VR-1 to regulate the internal levels of calcium. Additionally, the neuron uses calcium-binding proteins to chelate calcium in the cell during overload or toxic concentrations in order to prevent continuous stimulation and begin cellular recovery (reviewed by (2)).

During the exploration of the calcium unifying hypothesis, I noted that many of the same stimuli that cause reactivation in our latency model also modulate gating properties of the VR-1 channel. For example, PKC is known to regulate ion channels via phosphorylation and is also known to regulate IP3 channels in the endoplasmic reticulum, a calcium storage organelle. Vellani et al., (2001) determined that PKC activation in DRG cultures increased the probability of VR-1 channel opening (12). These investigators also demonstrated that VR-1 transiently transfected HEK-293 cells displayed an inward current upon exposure to the pharmacological agent, PMA. This

current could be abolished following capsazepine treatment, the specific inhibitor of VR-1. These studies suggest that PKC is involved in VR-1 gating and calcium modulation.

Another potent reactivation agent is the plant-derived substance, forskolin. Forskolin activates PKA via adenylate cyclase/cAMP causing a robust reactivation in the neuronal latency model that also involves alterations in VR-1 excitation. Because reactivation is robust and reliable following forskolin treatment, we often use it as a positive control when testing other compounds in the model. PKA and other agents that cause increased intracellular cyclic AMP levels pontentiate the VR-1 channel according to De Petrocellis et al., (2001) (6). Although most of these experiments were performed in VR-1 transiently transfected HEK-293 cells, the authors also tested ex-vivo rat DRG slices. They concluded that forskolin enhanced the effects of VR-1 activation and also increased substance-P release. Substance P excites nociceptor neurons and is normally present during tissue damage. These studies may imply that reactivation of HSV-1 is induced by overloaded calcium stimulation leading to neuronal modulation and changes in plasticity in the neuron. Our studies confirm these findings because forskolin-induced reactivation was severely attenuated and almost abolished following treatment with BAPTA (a calcium chelator) or capsazepine (VR-1 inhibitor). These studies also show that phosphorylation of CREB is not the only component necessary for reactivation and that calcium plays a pivotal role.

Other indirect evidence supporting the calcium unifying hypothesis is the role of calcineurin during HSV-1 reactivation. Calcineurin is a calcium/calmodulin dependent phosphatase whose role in the nervous system is not clear. Real time RT-PCR analysis of DRG total RNA detected an 11-fold increase in calcineurin following reactivation with

NGF deprivation (Mellenium Pharmaceuticals, Boston, MA). Calcineurin was one of the few RNA messages that increased significantly following reactivation with NGF deprivation. We examined various effects of calcineurin inhibitors (FK-503 and cyclosporin-A) in our latency model and their effects on reactivation. We could not correlate reactivation with inhibition of calcineurin. Following careful analysis of only the neuronal calcineurin literature, we learned that calcinuerin regulates calcium channels. A short communication article discussed the co-localization of calcineurin in DRG neurons with voltage-dependent calcium channels (VDCC) (9). Furthermore, adenovirus over-expression of calcineurin predisposed cortical neurons and PC12 cells to apoptosis (1). A more recent study by Vellani et al., (2001) discussed a model for calcineurin as a phosphatase that actually regulates gating of VR-1 (12). These authors hypothesize that calcineurin actually dephosphorylates VR-1, closing the channel to decrease calcium entry as a neuroprotective mechanism. Although there is no direct evidence that the VR-1 channel is gated via phosphorylation, the data suggest that this may be the case. Calcineurin activation during a neuronal insult fosters recovery from a potential calcium overload by deactivating the VR-1 channel.

Interestingly, release of calcium via internal calcium stores is not sufficient to induce HSV-1 reactivation in our model. Using ER poisons such as thapsigargin or cyclopiazonic acid had no effect on HSV-1 reactivation. There are two possible explanations behind these results. First, these internal calcium releasers do not increase calcium levels sufficiently to excite the neuron. Second, the neuron is able to recover from the calcium release by chelating the excess calcium using calcium-binding proteins or actively pumping calcium out of the cell. Studies in mouse DRG neurons by Shigmol

et al., (1995) showed that thapsigargin treatment following caffeine and high potassium solution abolished calcium currents (11). This study supports our conclusion that the DRG neuron can easily compensate for the calcium leakage from internal calcium store and that the calcium stores in DRG neuron are quite small.

Calcium imaging could directly address whether internal calcium concentrations are pivotal for reactivation of latent HSV-1. Using a recombinant virus that contains immediate early genes tagged with green fluorescent protein, we can alter intracellular calcium levels and determine whether particular calcium concentrations are important for HSV-1 reactivation. Furthermore, caged calcium experiments could determine the critical intracellular concentration necessary for reactivation of latent HSV-1.

The role of cAMP in calcium regulation and HSV-1 reactivation

Why are cyclic AMP agonists important regulatory signals for HSV-1 reactivation? Cyclic AMP is another signaling molecule with very diverse effects depending on the cell type and development stage. Stimuli that caused increases in cAMP also caused herpes reactivation (Table 5.1). More importantly, cAMP signaling is dependent on calcium.

Following neuronal maturation and differentiation, cAMP intracellular fluctuations may activate stress pathways. Upon neuronal maturation, increased levels of cAMP become a stress response stimulus as opposed to a survival stimulus. The neuron is no longer expressing the appropriate proteins and channels necessary for these levels of cAMP and undergoes apoptosis and begins to shut down. This may explain our observation that pharmacological agents that cause up-regulation of cAMP also induced reactivation in our *in vitro* model (Table 5.1). Surprisingly, overexpression of the

inhibitor to the cAMP response element (CRE) ICER, also caused reactivation in our model (5). These data suggest that ICER is involved in the regulation of the LAT promoter and necessary for the maintenance of latent infection. Furthermore, cAMP is necessary for maintaining neuronal survival and basal protein levels are required for regulating and continuously expressing survival gene products that contain the CRE regulatory element in addition to maintaining latency.

In addition to the role of cAMP in cell survival, this second messenger can regulate calcium levels and channel gating properties. These channels can cause altered states of depolarization. During neuronal development, the immature neuron relies on depolarization as a survival signal. The electrical activity of the neuron can influence gene regulation. Buonanno and Fields et al., (1999) discuss the differences in gene transcription and regulation following different types of electrical stimulus in the DRG (4). For example, burst timing stimulus, where the stimulus is applied with short delays between stimuli, can cause low transcription from the CRE element whereas, burst timing with longer delays between stimulus actually increases activity of the CRE element. Burst timing stimulus increases levels of calcium, increasing calcinuerin activity in the cell, whereas burst timing with longer delays decreases calcineurin and prolongs calcium increases and mediates high transcription from the CRE.

If electrical activity in the neuron changes gene expression via cAMP or calcium regulation, then hyperexcitation of a nociceptor *in vivo* may also have the same effects. These electrical stimulation experiments describing alternate activation of cAMP and regulation of calcium by Buonanno and Fields correlate with the data obtained on HSV-1 reactivation in our neuronal model, tying together the idea that excitation states can

change gene transcription via calcium and coincidentally result in herpes reactivation. First, we noted that stimuli that caused prolonged increases in intracellular calcium induced reactivation. Second, calcineurin is activated perhaps as a rescue signal during these prolonged calcium increases. Third, all the cAMP agonists tested initiated HSV-1 reactivation. Fourth, activation of VR-1 through heat or capsaicin stimulation initiated herpes reactivation that was reversible with the antagonist to the VR-1 calcium channel. Therefore, the role of calcium in herpes reactivation is not trivial and probably due to hyperexcitation of the nociceptor via calcium surges.

Capsaicin desensitization

But why don't we reactivate every time we eat that hot jalapeno at the Mexican restaurant? Well it lies in one very important aspect of the VR-1 channel, which is the fact that VR-1 is prone to desensitization following increased concentrations of capsaicin. Why would we express receptors that respond to the active component of hot chili peppers and our sensation is heat. We didn't evolve with these receptors solely for the purpose of enjoying jalapenos. These receptors are essential for painful heat sensation and harmful pH levels. Therefore, the serendipitous reason we respond to capsaicin is unique. For example, birds have evolved an insensitivity to capsaicin allowing them to spread the seed of the capsaicin containing plant (8). They may have different isoforms of the VR-1 receptor lacking the binding domains for capsaicin.

The active component in hot chili peppers binds in mysterious ways to the intracellular region of the VR-1 receptor and possibly at multiple sites. Perhaps capsaicin occupation of all binding domains saturates the receptor causing channel closure and

inactivation. This phenomenon has been shown in DRG cultures *in vitro*. Desensitization of the channel can be altered with different stimuli. Some electrophysiologists believe that VR-1 desensitization is due to intracellular calcium levels, whereas other investigators argue that desensitization is due to a voltage-gated event. In our experiments, we noted that reactivation with capsaicin occurred only at lower concentrations, perhaps indicating that the channel closed at the higher concentrations creating reduced intracellular calcium levels not conducive for herpes reactivation.

Conclusion

Sensory neurons are highly specialized polar cells that harbor latent HSV-1. The neuron more than likely establishes latency because sequential events essential for herpes replication are interrupted. In response, the virus remains as an episome in the nucleus of the dorsal root and trigeminal ganglia. Serendipitous host cellular signaling involving calcium stimulation causes increases in cellular transcription factors to initiate viral immediate early gene transcription. Likewise, possible release of a repressor element may also initiate transcription of IE and subsequent herpes replication and reactivation. Calcium levels necessary for pain perception are probably the culprits to this reactivation event. Further studies with more sophisticated calcium imaging may unravel some of the darkest secrets of the enemy that lie within.

Figure 5.1 HSV-1 Reactivation Signaling Model

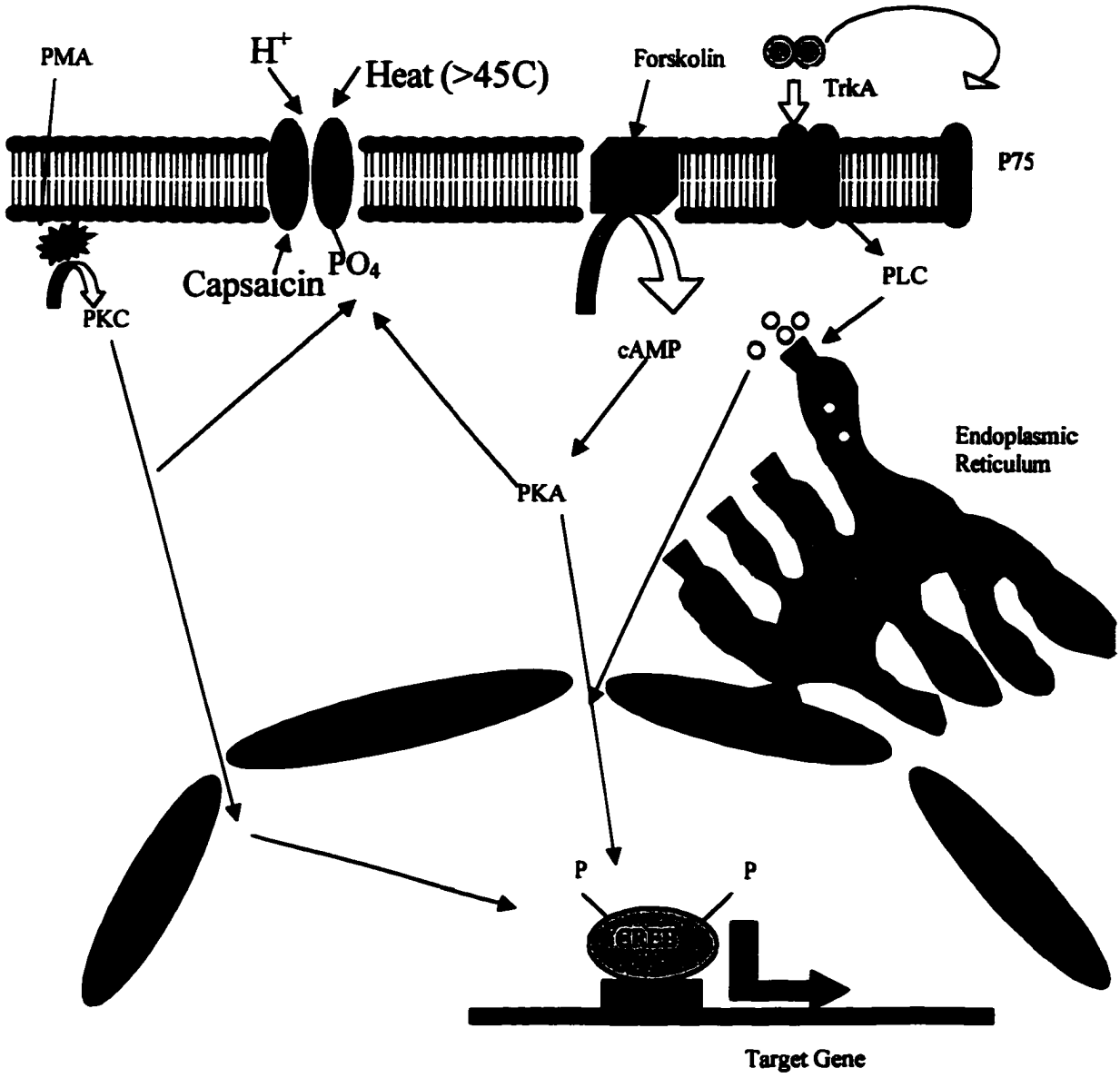

Table 5.1

HSV-1 Reactivation Stimuli	Secondary Pathways Associated with the Reactivation Stimuli
NGF Deprivation + anti-NGF treatment	Decrease in MAPK Decrease in AKT Turns on p75-JNK-p53-BAX
PMA 10^{-9-11}	Increase in PKC Activation of DAG
Forskolin 100μM	Increase in Adenylate Cyclase Increase in intracellular PKA Inhibits Phosphodiesterases
CPT-cAMP 500μM	cAMP agonist
IBMX 1mM	cAMP agonist
Capsaicin 0.1μM	VR-1 agonist Increases intracellular calcium
Heat Shock 42-46°C	Numerous pathways Activation of VR-1 between 45-46°C
Colchicine	Microtubule polymerization antagonist
Cyclohexamide	Protein synthesis inhibitor
Trichostatin A	Inhibitor of histone deacetylase

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