THE EFFECTS OF *HERPES SIMPLEX* VIRUS INFECTION ON VOLTAGE-GATED SODIUM CURRENTS IN ADULT RAT DORSAL ROOT GANGLION NEURONES *IN VITRO*.

by

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Abstract

*Herpes simplex* virus (HSV) is a common human pathogen which attains a lifelong latent state in sensory ganglia after initial infection at the periphery. HSV is a potential viral vector for neurological diseases. HSV is known, however to affect the electrophysiological properties of excitable cells *in vitro*. This study investigated the mechanism of electrophysiological changes that occur during an HSV infection. Studies of adult rat dorsal root ganglion (DRG) neurones in the whole-cell voltage-clamp mode showed that potassium and calcium voltage-gated currents were unaltered by wt HSV 17\(^+\) infection (wild type HSV Glasgow strain), whilst 70% of wt HSV 17\(^+\) infected DRG neurones had no sodium current after 24 hours. The tetrodotoxin sensitive and tetrodotoxin resistant sodium currents that remained in wt HSV 17\(^+\) infected DRG neurones after 24 hours were dramatically reduced in amplitude. The voltage dependence of these remaining sodium currents were unchanged.

Experiments with the protein synthesis inhibitor, cycloheximide, revealed that viral entry into DRG neurones was not sufficient to cause a loss of sodium conductance, and that viral protein synthesis was necessary. Infection of DRG neurones with HSV mutants lacking functional regulatory immediate early genes ICP0, ICP4 and ICP27 showed that their expression is necessary for the loss of sodium conductance. Incubation of wt HSV 17\(^+\) infected DRG neurones with the viral DNA replication
inhibitor, acyclovir, showed that viral DNA synthesis was necessary for the observed loss of sodium conductance.

A possible mechanism of loss of sodium conductance in wt HSV 17^+ infected DRG neurones is internalisation of sodium channels. The internalisation inhibitors, chloroquine, baflomycin A1 and ammonium chloride prevented the loss of sodium conductance in wt HSV 17^+ infected DRG neurones. A known trigger for internalisation of sodium channels from the surface of neurones is sodium influx. Treatment of DRG neurones with 50mM KCl and 0.1 mM veratridine, to evoke sodium entry, was sufficient to cause loss of sodium conductance. Preventing sodium entry during a wt HSV 17^+ infection by incubating cells either in sodium free media or with tetrodotoxin, was sufficient to prevent the virally induced loss of sodium conductance.
I would like to acknowledge my two supervisors Professor Stuart Bevan and Professor David Latchman for their continual support and guidance throughout my studentship. I feel especially indebted to Dr Linda McLatchie for her meticulous editing and support whilst writing this thesis. For swimming and spiritual guidance I was enlightened by Kirti Shah. I am truly grateful to Natasha Weeresekera, who is a wonderful friend to have. Particular thanks to Pamela Christman for sharing her “bird by bird” philosophy and many others that have saved me on innumerable occasions. For enthusiasm and nurture, I would like to recognise Virginia Vaughan and Angela Yphantides. Automatic thanks go to Sarah Aygekum who is the greatest dancer in the good times and whose understanding of the superstitions of the Ph.D. system dared me to survive. A nod and a wink goes to my brother for all his love. I dedicate this thesis to my parents with love.
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AMP  
adenosine monophosphate  
αTIF  
alpha trans-inducing factor  
ATP  
adenoine triphosphate  
BHK  
baby hamster kidney cells  
BSA  
bovine serum albumin  
CD8⁺  
cluster differentiation type 8⁺  
CNS  
central nervous system  
DHP  
dihydropyridine  
DMSO  
dimethyl sulphoxide  
DNA  
deoxyribose nucleic acid  
dpm  
disintegration per minute  
DRG  
dorsal root ganglion  
DTT  
dithithretol  
E  
potential  
EDTA  
ethylene diaminetetraacetic acid
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<th>Abbreviation</th>
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<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>$E_{\text{rev}}$</td>
<td>reversal potential</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>$g$</td>
<td>conductance</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>$h_\infty$</td>
<td>steady-state inactivation or availability parameter</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td><em>herpes simplex</em> virus</td>
</tr>
<tr>
<td>HVA</td>
<td>high-voltage activated</td>
</tr>
<tr>
<td>I</td>
<td>current</td>
</tr>
<tr>
<td>ICP</td>
<td>infected cell protein</td>
</tr>
<tr>
<td>IMF</td>
<td>isoleucine, methionine and phenylalanine</td>
</tr>
<tr>
<td>IR$_L$</td>
<td>inverted repeat long</td>
</tr>
<tr>
<td>IR$_S$</td>
<td>inverted repeat short</td>
</tr>
<tr>
<td>L</td>
<td>long</td>
</tr>
<tr>
<td>LAP</td>
<td>latency active promoters</td>
</tr>
<tr>
<td>LAT</td>
<td>latency associated transcript</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>LVA</td>
<td>low-voltage activated</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMLV</td>
<td>maloney murine leukemia virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribose nucleic acid</td>
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</table>
msec  milli second
NGF  nerve growth factor
Oct-1 octomer binding protein 1
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
pfu plaque forming unit
PKA protein kinase A
PKC protein kinase C
PMSF phenylmethylsulphonyl fluoride
PNS peripheral nervous system
S short
SDS sodium dodecyl sulphate
sem standard error of the mean
STX saxitoxin
syn syncytial
T thymus derived
TEA tetraethylammonium
TRIS Tris[hydroxymethyl]amino-methane
TR_L terminal repeat long
TR_S terminal repeat short
TTX tetrodotoxin
TTX-R tetrodotoxin resistant
TTX-S tetrodotoxin sensitive
U_L unique long
U_S unique short
UV ultra violet
V voltage
V_{1/2} or V_{50} voltage at half maximal
VHS virus host shutoff
V_{LI} junction potential
$V_m$ membrane potential

$V_{mw}$ virus protein molecular weight

$VP16$ virus protein 16

$wt\ HSV\ 17^+$ wild type Herpes simple virus strain $17^+$
Chapter 1

General Introduction

Summary of the aims and structure of the thesis

*Herpes simplex virus* (HSV) is a neurotropic virus that infects sensory neurones. Approximately 70% of people have been exposed to HSV-1, that causes predominantly facial cold sores, by their early twenties and by the time of old age almost everyone has been infected. HSV-2, that causes predominantly genital lesions, is a successful and ubiquitous pathogen and is the most common sexually transmitted disease (Ho, 1992). The virus, HSV-1, usually infects ocular and oral sites, although infection of cutaneous areas can occur particularly in immunocompromised people or via skin abrasions for example, many wrestlers
complain of cutaneous herpetic lesions (Selling & Kibrick, 1964). There is limited information about the effects that HSV has on infected neurones. The complaints of patients with HSV indicates very strongly that there are neuronal abnormalities during an infection. During the onset of recurrent infections people experience abnormal tingling sensations (paraesthesia), impaired sensations particularly of touch (dysesthesia), the feeling of insects crawling under their skin (formication) and a decrease in pain sensations (hypoalgesia). People complain of burning, shooting and boring pains that can last for many months after the lesions have healed (post herpetic neuralgia).

The aim of this study was to investigate the electrophysiological changes that occur during a replicative infection of HSV in DRG neurones. Previous studies have shown that the action potential of infected DRG neurones became smaller in amplitude and longer in duration and some neurones lost the ability to fire an action potential at all (Fukuda & Kurata, 1981). In this study the ionic currents underlying the action potential were investigated by the patch-clamp technique in the voltage-clamp mode in order to elucidate the cause of the altered excitability. The mechanisms by which these ionic currents changed during an HSV infection was investigated.

The pathogenic phenomenon of altered excitability during a HSV infection is of interest, not simply because it is fascinating to understand the pathology of herpes infections, but also because HSV has been developed as a potential gene therapy vector to deliver therapeutic genes for neurological disorders. It is of paramount importance that the delivery vector of the therapeutic genes does not render the neurones unexcitable, as this would be an unacceptable side effect of gene therapy treatments. This study investigated the pathological, electrophysiological changes during a wild type HSV infection. In addition, DRG neurones infected with HSV based gene therapy vectors were studied to examine whether there were any potentially damaging electrophysiological side effects from delivering foreign genes in this manner.
The thesis is split into 7 chapters beginning with a general introduction. This chapter introduces established ideas about the pathology of wt HSV 17^ infections in DRG neurones and gives an overview of the natural biology of HSV and the physiology of DRG neurones. The second chapter describes the materials and methods used in the experiments described in chapters 3-6. The voltage protocols and solutions used to record the ionic current changes that underlie the changes in excitability and action potential shape change are described. In addition to this, the techniques used to show that DRG neurones were infected with HSV and for growth of virus are explained. The results of experiments investigating which ionic currents were affected by a HSV infection are described in results chapter 3. In chapter 4, the life cycle of HSV was biochemically separated to investigate which aspect of viral infection caused the loss of excitability. Chapter 5 explores the possible cellular mechanisms underlying the loss of excitability and finally chapter 6 shows results of experiments investigating the effects that HSV based vectors had on DRG neurones. The final discussion chapter summarises the findings of this study and discusses their relevance in terms of viral pathogenesis and gene therapy.

**General introduction**

**The mystery of Herpes simplex virus**

Genetic evidence suggests that Herpes viruses have infected people since ancient times (McGeoch et al. 1995). Thousands of years ago Neolithic people were known to live in small groups of about 30-60, who were geographically separated from other groups by valleys, forests and rivers. Viruses either kill their host or are eradicated by the host immune response. Thus, viruses can only survive by jumping from an infected to an uninfected, immuno-naïve individual. A population of about 200 000 would be required to support a herpes-like virus. This presents a paradox, however, as an outbreak of herpes virus in a small population, such as the Neolithic group, would
have infected all susceptible individuals within a couple of weeks and the virus would have disappeared forever (Johnson, 1998).

In fact, the actual cause of cutaneous lesions or common cold sores was a mystery until as recently as the turn of the 19th century. Scientists believed that the herpetic (from the Greek herpein “to creep”) lesions were produced endogenously and not caused by an infectious agent as the sores recurred in the absence of a new infection. These ideas were proved wrong, however, after a series of experiments showed that the fluid from a lesion could be used to inoculate either the same person again or produce herpetic keratitis in rabbits (Stevens, 1975). These findings demonstrated that there was in fact an infectious causative agent now known as Herpes simplex virus (HSV). The mystery still remained, however, as to how the herpetic lesions could repeatedly reappear at the initial site of infection without any re-exposure to the virus.

A neurosurgeon, Harvey Cushing, made some pivotal observations whilst trying to relieve patients of trigeminal neuralgia by cutting their trigeminal nerves. He noticed that two patients developed herpetic lesions in mucosal areas supplied by the intact nerve but not by the cut nerve. From these observations, he concluded that the trigeminal nerve must be intact in order for the development of lesions and that the nerve cell bodies located in the trigeminal ganglia must be involved since they are common to all the branches of the trigeminal nerve (Cushing, 1905). These observations were extended by Paine (1964) who found that cutting the trigeminal nerve very close to the ganglia or destroying the ganglia by injection of alcohol prevented the appearance of lesions. This lent further support to the idea that the cell bodies in the sensory ganglia played a role in the appearance of the herpetic lesions. Some years later, experiments with HSV infected mice showed that virus could be recovered from explanted ganglia but not from the remaining nerve trunks. The reactivated virus was detected in the culture media of the explanted ganglia by formation of plaques on a lawn of permissive cells, or detected directly in the ganglia by in situ nucleic acid hybridization (Stevens & Cook, 1974). (A plaque is an area of lysis or a hole formed in a lawn or confluent monolayer of cells due to infection with an infectious unit of cytopathic virus.) Thus, Cushing’s original ideas were proved to
be correct; the virus forms a latent infection in the cell bodies of sensory neurones, which form an apparent reservoir of virus for secondary infections at the original site of infection where the nerve terminates.

It is the ability of herpes viruses to form latent infections that solves the puzzle of herpes virus survival in Neolithic human beings. After an acute infection many years may pass before the reactivation of virus. This can initiate a new epidemic within a new generation which has no immunological protection against the virus.

Natural biology of *Herpes simplex* virus

*Herpes simplex* virus belongs to the family of Herpesviridae. Members of this virus family are characterised by viral architecture. Specifically they contain single, linear, double stranded DNA genomes packaged in icosahedral nucleocaspids of about 100nM diameter. The nucleocaspids are surrounded by an amorphous material known as tegument. The virus is further enclosed by a lipid envelope which is derived from the host. The envelope contains viral glycoproteins that are involved in cell entry. There are over 100 known members of the Herpesviridae family, most of which are restricted to their natural hosts. The eight human herpes viruses characterised at present are summarised in table 1.1 below.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Latency</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes simplex -1</td>
<td>sensory ganglia</td>
<td>cold sores of oral mucosal membranes, encephalitis and Bell's palsy</td>
</tr>
<tr>
<td>Herpes simplex -2</td>
<td>sensory ganglia</td>
<td>Herpes genitalis and encephalitis</td>
</tr>
<tr>
<td>Varicella zoster</td>
<td>sensory ganglia</td>
<td>chicken pox and upon reactivation, shingles</td>
</tr>
<tr>
<td>Epstein-Barr</td>
<td>hematogenous cells (B cells)</td>
<td>infectious mononucleosis, meningitis, encephalitis and Burkitt lymphoma</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>B cells</td>
<td>infectious mononucleosis and encephalitis</td>
</tr>
<tr>
<td>Human herpesviruses 6 and 7</td>
<td>T cells</td>
<td>meningitis or encephalitis</td>
</tr>
<tr>
<td>Human herpes virus 8</td>
<td>lymphocytes</td>
<td>Kaposi’s sarcoma</td>
</tr>
</tbody>
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Table 1.1. The diseases caused by human herpes viruses and the cells that harbour the latent infection.

Herpes viruses can be further sub-classified into the alpha-herpesviridae based on their relatively short replication cycle, ability to destroy the infected cells of the epidermis, neurotropism and establishment of latency. Included in the alpha-herpesviridae sub group are *Herpes simplex* virus type 1 and 2, *Varicella zoster* and *Pseudorabies* virus.

**Virion structure**

The HSV virion consists of 4 components: linear, double stranded DNA encased in the 100 nM diameter icosahedral capsid made of 162 capsomer proteins, tegument and an outer envelope (Roizman & Sears, 1996). An electron micrograph and schematic representation of the HSV virion is shown in figure 1.1.
The complete nucleotide sequence of the HSV genome has been determined (McGeoch et al. 1988). It is approximately 152kbp long and has a 68% C and G content. It consists of two covalently linked unique regions known as L (long 126kbp) and S (short 13kbp) flanked by inverted repeats which allows for the L and S components to invert relative to each other and form four isoforms that are present in equal proportions. Mutations designed to prevent isomerisation do not seem to disadvantage replication and the biological relevance of this isomerism is unknown (Jenkins & Roizman, 1986). The HSV-1 genome contains at least 72 potential protein coding genes (McGeoch et al. 1988). Figure 1.2 shows a schematic diagram of the HSV-1 genome showing the internal and terminal repeat sequences. The positions of the immediate early genes, the first genes transcribed in the lytic cycle are shown and labeled by the name of the protein.
Figure 1.2. A simplified map of the HSV-1 genome and the positions and orientations of the genes which encode for immediate early infected cell proteins (ICP). The internal and terminal repeat sequences are labeled TR and IR, the subscript denotes which unique (U) segment they are associated with long (L) or short (S).

Life cycle

Lytic infection

The processes underlying the lytic phase of the viral life cycle, during which the virus replicates, are complex. The key events have been summarised in figure 1.3. A more in-depth discussion of the features of the cycle that are of particular relevance to this study follows.
Figure 1.3. A schematic representation of the HSV replication cycle. a: The virus attaches to the cell surface and penetrates into the cytoplasm by fusion of viral and plasma membranes. b: The viral DNA circularises in the nucleus and the first set of viral genes, the immediate early genes, are transactivated by the virion tegument protein ICP25 (Vmw65 or VP16) and cellular factor Oct-1. c: The immediate early proteins regulate the expression of the early and late genes. d: The second set of proteins are synthesised; the early proteins. These, in turn, initiate the transcription of the last set of genes. e: The early proteins are mainly involved in viral DNA replication which occurs by the rolling circle mechanism. f: The last wave of proteins synthesised are the late proteins which are structural proteins necessary for the assembly of the virion particle. g: The capsids are assembled and bud from the nuclear membrane. h: New virions are transported through the cytoplasm in transport-like vesicles which fuse with the plasma membrane as the virions egress into the extracellular space.
The first step in viral invasion of cells is the attachment of HSV-1 glycoproteins to cell surface heparan sulphates (Feyzi et al. 1997; Shieh & Spear, 1994). After attachment to the cell, the viral envelope fuses with the cell plasma membrane. This process is also mediated by the viral glycoproteins (Campadelli-Fiume et al. 1988; Morgan et al. 1968). Once inside the cell, the nucleocapsids are rapidly transported along microtubules across the cytosol to the nuclear pores where the viral DNA is released (Batterson et al. 1983; Sodeik et al. 1997).

HSV-1 gene expression is tightly regulated. Three classes of viral genes, the immediate early, early and late viral genes are sequentially expressed in a defined temporal cascade (Honess & Roizman, 1974). Viral genes are expressed at very high levels whilst cellular genes are greatly suppressed. Two of the tegument proteins, which are delivered to the cytoplasm after fusion of host and viral membranes, play an important role in the switch from host to viral gene expression. They are the virus host shut-off protein (vhs) and ICP25 (also known as VP16, Vmw65 and α trans inducing factor or αTIF).

The crucially placed tegument protein vhs can influence early events in the HSV replication. The protein, vhs, which is encoded by gene U₄₁, shuts off host protein synthesis by accelerating the turnover of preexisting cellular mRNAs and disrupting polyribosomes (Fenwick & McMenamin, 1984; Fenwick & Clark, 1982; Read et al. 1993). The molecular mechanism of the mRNA disruption is unknown, although vhs may target proteins associated with RNA or have an endolytic function (Strom & Frenkel, 1987). The degradation of mRNA is indiscriminate, such that vhs does not distinguish between viral and cellular mRNAs. If it was not for the fact that the function of vhs is inhibited during the HSV replication cycle, then the viral mRNA would not persist (Fenwick & Everett, 1990). Results from two experiments suggested that the tegument protein ICP25 was a likely candidate for the protein which dampens down the function of vhs in order to allow the accumulation of viral mRNAs. Firstly, cells infected with HSV viruses with a null mutation in the gene encoding ICP25 had unstable viral mRNA which resulted in reduced viral protein synthesis.
Secondly, cells that constitutively expressed the gene encoding ICP25 did not show protein synthesis shut-off when infected with wild type virus (Lam et al. 1996).

The tegument protein ICP25 has several other crucial functions, in addition to rescuing HSV from its own protein synthesis inhibitor. Deletion mutation of the gene encoding for ICP25 is lethal as new virions fail to be packaged. This is because ICP25 is an essential structural component of the virion (Weinheimer et al. 1992). ICP25 also has a critical role in activating transcription of the first set of viral genes to be expressed, the immediate early genes (Batterson & Roizman, 1983). This was discovered by an elegant set of experiments in which a chimeric thymidine kinase gene under the control of the immediate early gene promoters was inserted in the viral genome. This virus infected cells in the presence of the protein synthesis inhibitors, cycloheximide and these cells were then superinfected by a virus with a deleted thymidine kinase gene. Due to the presence of cycloheximide, no viral protein synthesis occurred. However, thymidine kinase mRNA still built up in the cells, showing that it was a virion component that induced the transcription of the thymidine kinase gene under the immediate early gene promoter (Post et al. 1981). The trans-activating factor ICP25 has little intrinsic DNA binding activity in isolation but was found to bind to DNA strongly in the presence of a ubiquitous cellular transcription factor Oct-1 (Ace et al. 1988; O'Hare & Goding, 1988; McKnight et al. 1987). The Oct-1 and ICP25 regulatory complex recognises the region of the immediate early gene promoter with the sequence TAATGARAT and due to the strong acidic transactivation domain of ICP25, strongly activates immediate early gene expression (Gaffney et al. 1985). The trans-activating factor ICP25 is non essential for viral replication. Mutations that prevent the binding of ICP25 to Oct-1 and consequently result in loss of the ability to trans-activate their immediate early genes are not lethal to the virus. Viruses containing these mutations do, however, show impaired ability to multiply and spread at low multiplicity (Ace et al. 1989).

Large amounts of ICP25 are produced late in infection, yet transactivation of viral immediate early genes is not observed. This puzzle is solved by the fact that newly synthesised ICP25 forms a complex with vhs and is packaged into virions away from HSV DNA. The fact that vhs and ICP25 interact solves another important question,
i.e. why does vhs not destroy viral mRNA? During the initial stages of infection ICP25 and vhs perform their functions in different compartments of the cell and thus do not interfere with each other's functions.

In summary, the functions of the tegument proteins vhs and ICP25 confer a swift switch between host cell and viral protein synthesis. Vhs disrupts the host cell protein synthesis leaving the molecular machinery free for viral mRNA translation, whilst ICP25 together with Oct-1, rapidly and strongly activates the transcription of the five viral immediate early genes.

Between 2-5 hours post infection, the first genes to be transcribed in the cascade of viral gene expression are the five immediate early genes (also known as α genes) that encode ICP0, ICP4, ICP27, ICP22 and ICP47 (Pereira et al. 1977; Honess & Roizman, 1974). With the exception of ICP47, these immediate early genes contribute to the regulation of expression of viral functions at the transcriptional and post transcriptional level. ICP4 and ICP27 are essential for viral growth as they have a regulatory function necessary for activation of subsequent kinetic sets of genes in the cascade. ICP4 can also autoregulate its own expression. ICP0 is not essential for viral growth, but does enhance efficient viral replication, as mutants lacking ICP0 grow very sluggishly. ICP22 also has a regulatory function, although it is non essential for viral replication and mutant viruses lacking ICP22 show a delayed shutoff of the early genes in addition to reduced late gene expression and capsid formation (Sears et al. 1985). ICP22 also down regulates the expression of viral immediate early genes (Prod-hon et al. 1996). A schematic representation of the role of immediate early genes in the temporally regulated cascade is shown in figure 1.4.
Figure 1.4. The role of the immediate early gene products ICP4, ICP27, ICP0 and ICP22 on the temporal expression of viral genes. This schematic diagram illustrates the regulatory effects of immediate early genes showing the positive (+) and negative (-) effects that these transactivators can exact on early and late gene transcription. The tegument protein ICP25, which enters the cell after adsorption, recruits cellular transcription factor Oct-1 to form a complex which transactivates the expression of the viral immediate early genes. This diagram is modified from (Glorioso et al. 1995).

The immediate early gene which does not have a regulatory function, ICP47 was recently found to have a role in protecting the HSV infected cell from the immune response of the host. CD8$^+$ T lymphocytes, (cytotoxic T cells) recognise peptide antigen presented on the cell surface with the major histocompatibility complex (MHC) class 1 molecules and destroys the cell by lysis. The resistance of HSV infected cells to this lysis is conferred by immediate early gene ICP47 (York et al. 1994; Posavad et al. 1993). In uninfected cells, the peptide antigens from the cytosol are transported across the ER membrane by a molecule known as transporter associated with antigen processing (TAP) (Neefjes et al. 1993). ICP47 prevents substrates from binding to TAP, which prevents the assembly of antigenic peptides with MHC class 1 and ultimately averts the recognition of infected cells by cytotoxic T cells.

Between 5 and 7 hours after infection, the early genes (also known as β genes) are at their peak rate of expression. The early genes encode for proteins which are mostly
involved in DNA metabolism and replication. HSV DNA circularises immediately after infection and replicates by the rolling circle mechanism which generates concatamers that are cleaved and packaged into the capsid forming nucleocapsids (Ben Porat & Tokazewski, 1977). The virus encodes for some enzymes which are necessary for DNA replication such as ICP8 which destabilizes and unwinds double stranded DNA, and 5'-3' helicase, whilst hijacking some host enzymes such as DNA polymerase and ligase 1 (Boehmer & Lehman, 1997; Boehmer & Lehman, 1993).

The late genes (also known as γ genes) begin to be expressed after about 10 hours of infection. There are 48 late genes which mainly encode for structural proteins such as glycoproteins, capsomer proteins, and tegument proteins like ICP25. The late genes can be further subdivided on the basis of their requirement for viral DNA synthesis for their expression. The “leaky” late genes are only slightly affected by inhibition of viral DNA synthesis, whereas the “true” late genes expression are not expressed at all when viral DNA synthesis is inhibited (Holland et al. 1980).

The capsids are assembled and DNA is packaged into them in the nucleus. Then the nucleocapsid buds from the nucleus gaining an envelope in the process. In sensory neurones this is seen to occur, as judged by electron microscopy, at about 20 hours post infection (Lycke et al. 1988). Further envelopment may occur at the ER, and the virion may pass through the Golgi apparatus although this is not certain. Certainly the virions are present in the cytoplasm enclosed in membrane bound transport-like vesicles (Schwartz & Roizman, 1969). The virions are released into the extracellular space when the transport-like vesicle fuse with the plasma membrane (Roizman & Sears, 1996).

The commonly used pharmacological agent that prevents recurrent episodes of cold sores is acyclovir. It is a guanine derivative (acycloguanosine) that acts by inhibiting HSV DNA polymerase and thus preventing viral DNA replication. Acyclovir is phosphorylated by viral thymidine kinase and is subsequently able to inhibit DNA replication. In uninfected cells, acyclovir does not become phosphorylated and even if it were to become phosphorylated it inhibits cellular DNA polymerase 30 times less
efficiently than the viral polymerase. It is these characteristics which make acyclovir a very selective drug that inhibits viral and not host DNA synthesis (Elion et al. 1977).

**Latency**

A characteristic feature of HSV infections is the ability of the virus to form latent infections. Following the initial infection the virus travels by axonal transport to the cell body situated in the dorsal root ganglion where the virus is maintained in the latent state (Carter et al. 1992). The virus can be periodically reactivated to produce a secondary episode of cold sores. The reactivated virus travels back to the tissues again by axonal transport and initiates a lytic infection at the original site of infection. The molecular mechanism of viral latency in sensory cell bodies hinges upon the transactivation of immediate early genes by tegument protein ICP25 and the ubiquitous, cellular transcription factor Oct-1. Oct-1 is present in sensory neurones but other transcription factors, Oct-2.4 and Oct-2.5 are more abundant (Kemp et al. 1990). ICP25 interacts with Oct-1 at the POU homeodomain, a conserved region of about 60 amino acids that bind specific gene sequences. Oct-1 and Oct-2 homeodomains differ at only 7 positions and it is this difference that allows ICP25 to discriminate between them (Stern et al. 1989). In sensory neurones Oct 2.4 or Oct-2.5 binds to the TAATGARAT sequence on immediate early gene promoters and consequently blocks the binding of the transactivating complex Oct-1/ICP25. Under these conditions the immediate early genes are not expressed and thus the lytic replication cycle never begins. The only viral transcription known to occur during latency is that of the latency associated transcripts (LATs) although no protein related to these transcripts has ever been found (Ho, 1992). The function of the LAT transcripts remains debatable. Lytic infection will occur in neurones when there are large quantities of virus, as the block of immediate early gene transcription is overridden.
Gene therapy

The biology of HSV makes it a very attractive candidate for a vector to deliver therapeutic genes to diseased neurones. HSV infects neurones, establishing a life long presence as an episome during which time LATs are transcribed continually (Mellerick & Fraser, 1987). The possibility of exploiting HSV to deliver foreign genes to neurones with the option of long term transgene expression using latency active promoters seems logical. Another advantage of HSV as a potential gene therapy vector is the fact that the very large genome can be divided roughly in half based on whether the genes are essential for viral replication or not. Hence, large foreign genes can be inserted into the genome without rendering the virus defective for growth. The virus also grows well in culture so that high titre stocks of a gene therapy vector could be made. There is, however, a problem with cytotoxicity. HSV has profound effects on the cells it infects and in permissive cells will ultimately destroy the cell by lysis. Attempts to make safe viral vectors have been made by preventing the virus from replicating by removal of immediate early genes, and designing cell lines to complement these genes in trans for viral growth. Although much more research has to be done in order to achieve a safe viral vector for gene therapy the potential is there to design vectors for the therapies for diseases such as Parkinson’s or Alzheimer’s.
Dorsal root ganglion neurones

HSV forms latent infections in the cell bodies of sensory neurones that are located in the dorsal root. The role of the DRG neurones is to convey sensory information about temperature, position or harmful stimuli, from the periphery to the central nervous system. With this direct connection to the periphery the dorsal root ganglion is the perfect place for a virus such as HSV to lie dormant whilst waiting for a second opportunity to infect the periphery. This positioning also explains why the HSV virus is able to migrate by inter neuronal spread to the central nervous system causing fatal encephalitis. The spinal sensory neurones were first described as emerging from the dorsal root by Charles Bell (1811). He described that the afferent (sensory) fibers had their cell bodies in the dorsal root and it is for this reason that they were called dorsal root ganglion (DRG) neurones (Perl, 1992).

These neurones are a heterogeneous group that vary in many respects such as biochemical make up, developmental connections and membrane properties. An intrinsic property of these neurones is the rate at which they conduct an action potential, known as conduction velocity. When the whole peripheral nerve is stimulated the compound action potential shows a series of waves which is the result of input from groups of fibres with different conduction velocities (Gasser, 1938). The fibres with the fastest conduction velocities were called the A fibres (>1.3m/s) and can be further sub divided into clearly distinct groups which were labeled α (>12m/s), β (30-100m/s) and δ (4-30m/s) from fastest to slowest conduction velocities. The groups of fibres that had much slower conduction velocities were called C fibres (<1.3m/s) (Meyer et al. 1994). The conduction velocity was found to correlate strongly with the diameter and degree of myelination of the neurones (Gasser & Erlanger, 1927; Harper & Lawson, 1985). About 30% of the DRG neurones population conduct with a fast conduction velocity (Aα, Aβ or Aδ neurones), these neurones are myelinated and large in diameter. In contrast, the more slowly conducting C fibres are smaller in diameter and are non-myelinated and make up about 70% of the DRG neurone population (Yoshimura et al. 1998).
Dissociated DRG neurones can also be separated into sub populations on the basis of their morphology. Dark neurones, which have a dense distribution of organelles, are predominantly small in somal diameter, whilst light neurones that contain large amounts of neurofilament have a wider diameter range (Lawson et al. 1984). It is also possible to sub-divide the DRG neurone population on the basis of the biochemical composition of DRG neurones which can be correlated with neurone morphology or conduction velocity. For example, small dark DRG neurones that also have a slow conduction velocity have a lower neurofilament and carbonic anhydrase content, higher capsaicin sensitivity, high substance P content and lactoseries oligosaccarides on the cell surface when compared to the light neurones (Gold et al. 1996). Additionally, various markers and dyes can be used to identify populations of DRG neurones. For example, the I-B₄ isoelectin from the plant Bandeiraea simplicifolia binds specifically to α-D-galactose carbohydrate groups. A sub-population of DRG neurones that are of small diameter and have unmyelinated fibres selectively express the carbohydrate moieties that I-B₄ recognises. Thus, I-B₄ is used as a marker of some primary afferent nociceptors and can be used to show where these neurones terminate (Kitchener & Snow, 1993). Not all small DRG neurones are I-B₄ positive, however, as a newly characterised population of small DRG neurones that expresses the glia cell derived neuroptrophic factor (GDNF) receptor and very high levels of the vanilloid 1 receptor (VR1) mRNA is not I-B₄ positive (Micheal & Priestly, 1999).

The classification systems do cross as C and Aδ fibres have small dark cell bodies whilst Aα and Aβ fibres have light cell bodies (Harper & Lawson, 1985). DRG neurones have varied biochemical contents that correlates to their many different functions. For example, poly-modal nociceptors that transmit information about potentially harmful stimuli to the central nervous system, are either Aδ or C fibres with a small dark somas and are capsaicin sensitive.
Action potentials

Action potentials are the rapid electrical signals that are transmitted along axons. In the case of the DRG neurones, these signals transfer information from the periphery to the central nervous system. Early descriptions of the classic action potential shape came from the squid axon (Huxley & Hodgkin, 1952). The action potential shown below was evoked from a DRG neurone and shows the typical shape of an action potential.

![Image of action potential]

**Figure 1.5. Amplitude and time course of an action potential of a DRG neurone.**
The neurone was clamped in the current-clamp mode and the membrane voltage was recorded after an injection of 100pA. The pipette and bath solutions used mimicked the physiological surroundings and contents of the DRG neurone (see methods).

The action potential is produced by the coordinated activity of at least two different types of ion channels, the sodium channel and the potassium channel. Voltage-clamp recording revealed the effects of membrane potential on the sodium and potassium conductances that underlie the action potential. These experiments were, again, first performed on the squid axon (Hodgkin & Huxley, 1952). Since then advances first in two electrode voltage-clamp and then in whole-cell recordings with "patch electrodes" have allowed small neurones to be studied (Hamill et al. 1981). When the same DRG
neurone whose action potential is shown above in figure 1.5 was clamped in the voltage-clamp mode and the membrane depolarised to +20mV, the sodium and potassium current components were clearly distinguishable. Figure 1.6 shows the two major current components of the action potential.

Figure 1.6. Voltage-clamp recordings showing the effects of membrane potential on the sodium and potassium conductance. The membrane potential was held at -120mV and then stepped to +20mV. In response to this depolarisation, the sodium current activated allowing a sodium influx and then inactivated. The potassium current, that activates at a slower rate, allowed potassium ions to flow out of the DRG neurone. The composition of the pipette and bath solutions used were designed to represent the physiological environment (see methods).

An action potential is an “all or none” phenomenon that occurs when a membrane depolarisation activates an inward sodium ion flow that is greater than the outward current caused by the increased driving force on potassium ions. This potential at which the depolarisation becomes regenerative is known as the threshold potential and
simply represents the potential at which the inward current overrides the outward current.

Action potentials are initiated by the activation of voltage-sensitive sodium channels that mediate a rapid influx of sodium ions which depolarises the cell membrane from a resting membrane potential of about -60mV. The influx of the sodium ions depolarises the membrane in a positive feedback manner. As sodium ions enter the cell the membrane depolarises and consequently activates more and more sodium channels. Sodium conductance decreases as the action potential reaches its peak, however, because the sodium channels inactivate and no longer respond to depolarisation. The inactivation of the sodium channels contributes to the repolarisation stage of the action potential. The falling phase of the action potential is also achieved through the activity of the potassium channels that activate 10 times more slowly than the sodium channels and selectively allows the exit of potassium ions from the cell (Hille, 1992). The membrane potential is thus driven closer to the potassium equilibrium potential, which is about -90mV, and has the effect of repolarising the membrane potential.

Voltage-sensitive calcium ion conductance can also contribute to the shape of the action potentials in DRG neurones. The membrane potential depolarisation during the upstroke of the action potential activates the voltage-gated calcium channels. Many types of calcium channels do not inactivate and for this reason can give the action potential a prolonged duration and the appearance of a plateau or “hump” in the falling phase of the action potential.

The balance of these ion channel conductances governs the shape of the action potential, such as amplitude and width at half maximal voltage. Within the heterogeneous population of DRG neurones the shape of the action potential varies and can be grouped into neurone subtypes (Harper & Lawson, 1985). This is a reflection of the differential distribution of ion channels in DRG neurone sub-populations (Mandel, 1993). The sodium, calcium and potassium channels are members of gene families encoding for voltage-gated ion channel proteins.
Voltage-gated sodium channels are responsible for the generation of action potentials in most excitable cells. Sodium channels can be differentiated into two subtypes on the basis of their sensitivity to two marine toxins, tetrodotoxin or saxitoxin (TTX and STX). Tetrodotoxin is a poison from the puffer fish and other members of the Tetraodontidae family that blocks action potential conduction in nerves. Voltage-clamp studies on lobster axons showed that nanomolar TTX specifically blocks the sodium currents while the calcium and potassium conductances were unaffected (Narahashi et al. 1964). STX is a toxin produced by marine plankton that has very similar properties to TTX and blocks sodium channels at the same site (Hille, 1968).

The first evidence that there were more than one type of sodium channel in DRG neurones was obtained by observing the effects of TTX on action potential shape (Fukuda & Kameyama, 1980; Yoshida et al. 1978). The sodium channels of DRG neurones can be separated into two groups based on their sensitivity to TTX, one which was abolished by nanomolar concentrations of TTX, named the tetrodotoxin sensitive (TTX-S) channel and the other that was only half blocked by as much as 75 µM TTX, called the tetrodotoxin resistant (TTX-R) channel (Kostyuk et al. 1981; Roy & Narahashi, 1992). Voltage-clamp analysis of sodium currents in DRG neurones showed that the two channel currents had different biophysical properties. Figure 1.7 illustrates the differences between the TTX-S and the TTX-R sodium current types (see Elliott & Elliott, 1993; Roy & Narahashi, 1992).
Figure 1.7. A family of TTX-S and TTX-R sodium currents and the relationship between voltage and activation and inactivation of the sodium channels. 

a A family of TTX-S sodium currents obtained by subtracting the family of TTX-R sodium currents evoked after a prepulse to -50mV, from a family of total sodium currents evoked after a prepulse to -120mV. 

b A family of TTX-R sodium currents was evoked after a prepulse to -50mV to remove the inactivation from the TTX-R sodium channels but not the TTX-S sodium channels. 

c The relationship between command potential and conductance of TTX-S and TTX-R sodium channels. The TTX-S channels activate at about -50mV, whereas the TTX-S channels activate at about -30mV. 

d The relationship between the inactivation parameter $h_{in}$ and the prepulse potential. The TTX-S and TTX-R sodium channels have different inactivation properties such that at -50mV the TTX-R sodium channels are fully activated whilst the TTX-S channels remain fully inactivated.
Table 1.2 summarises the properties of the two different sodium current types, the TTX-S and the TTX-R sodium currents in terms of sensitivity to TTX, activation and inactivation kinetics and voltage at half maximal activation ($V_{50}$) and inactivation ($V_{\text{hoo}}$).

<table>
<thead>
<tr>
<th>Biophysical Properties</th>
<th>TTX-S</th>
<th>TTX-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$ of TTX</td>
<td>0.3nM</td>
<td>100μM</td>
</tr>
<tr>
<td>activation range</td>
<td>&gt;-60 mV</td>
<td>&gt;-40 mV</td>
</tr>
<tr>
<td>$V_{50}$</td>
<td>~30 mV</td>
<td>~15 mV</td>
</tr>
<tr>
<td>activation kinetics</td>
<td>fast (~5 msec)</td>
<td>slow (~10 msec)</td>
</tr>
<tr>
<td>$V_{\text{hoo}}$</td>
<td>~70 mV</td>
<td>~30 mV</td>
</tr>
<tr>
<td>inactivation kinetics</td>
<td>fast (~1.4 msec)</td>
<td>slow (~5 msec)</td>
</tr>
</tbody>
</table>

Table 1.2. The electrophysiological characteristics of TTX-S and TTX-R sodium currents. The difference of inactivation kinetics means that a prepulse to -120mV will remove the inactivation of all sodium channels, however a prepulse -50mV will inactivate the TTX-S sodium channels whilst the TTX-R channels are not inactivated.

In summary, there are two subtypes of sodium current present in DRG neurones, the low threshold, rapidly activating and rapidly inactivating current which is sensitive to low (nM) TTX, and the TTX-R current that is a higher threshold, slowly activating, slowly inactivating current that is resistant to TTX. The presence of TTX-R sodium channels with their slower inactivation kinetics in addition to calcium currents contribute to giving an action potential a longer duration (Yoshida et al. 1978).

The first sodium channel was cloned from the Eel electroplax, and the hydrophobicity plots were used to deduce the secondary structure of the channel in the plasma membrane (Noda et al. 1984). The $\alpha$ subunit is the actual pore of the channel and alone is sufficient for functional ion conductance. A schematic diagram of the sodium channels $\alpha$ subunit secondary structure is shown in figure 1.8. The heavily glycosylated $\alpha$ subunit was found to consist of 4 homologous domains (labeled I to IV) each with 6 putative membrane spanning $\alpha$ helical segments (labeled 1 to 6). The
(tertiary structure) 3 dimensional topography of the four domains are not linear in the membrane as depicted in figure 1.8, but fold around each other to form a pore.

![Diagram of voltage-gated sodium channel](image)

**Figure 1.8. The secondary structure of the voltage-gated sodium channel α subunit.**

The cylinders represent putative alpha helical segments. The activation/voltage sensors are the highly conserved, positively charged S4 segments. The puffer fish toxin, tetrodotoxin binds to the extracellular loop between segment 5 and 6 of domain I (Noda *et al.* 1989; Hille, 1968). The putative protein kinase A phosphorylation sites are shown as ○ whilst the protein kinase C site is labeled □.

In the sodium channels the extracellular linkers joining transmembrane segments 5 and 6 or P regions form the lining of the pore and confer ion selectivity to the channel. The activation of the ion channel controls the rate and voltage dependence of the channel opening. The general strategy of activation hinges on the fourth transmembrane segment 4 that moves in response to changes in membrane depolarisation and results in channel opening. The S4 segments of the channels contain positive charges (lysine or arginine) and are thought to be form the putative voltage sensor, and substitution of these positive amino acids results in non-activating channels (Stuhmer *et al.* 1989).

Inactivation causes the channel to close during a maintained depolarisation. Inactivation occurs spontaneously, presumably as a result of conformational changes during channel activation. The intracellular linker between domain III and IV migrates to the mouth of the inner vestibule of the pore and blocks conduction. The time course of this movement is such that there is enough time for sodium ion influx before

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inactivation and before potassium currents restore the membrane potential. The presence of proteases in the patch pipette destroys sodium channel inactivation. This is because the intracellular linker between domain III and IV, is cleaved off by the protease (Rojas et al. 1973). Veratridine, a poison from Lily species Veratrum, binds within the sodium channel pore (site 2) and prevents inactivation and so allows prolonged activation of sodium channels (Barnes & Hille, 1988). The loops connecting domains III and IV contain a critical IMF (isoleucine, methionine and phenylalanine) motif within the inactivation gate. Site directed antibodies to this linker region blocked inactivation and mutations within this cluster of three hydrophobic amino acids prevented inactivation (West et al. 1992). Small peptides containing IMF supplied in the patch pipette can restore the fast inactivation (Eaholtz et al. 1994). The connecting loops of segments 4 and 5 of each domain are located near the mouth of the intracellular side of the pore and mutagenesis studies have shown that they play a role in inactivation possibly by binding to the IMF motif during channel inactivation (McPhee et al. 1995).

TTX and STX bind to a site (site 1) which is located on the extracellular loop between S5 and S6 in domain 1. When the toxin is bound it blocks the pore preventing sodium flow without altering sodium channel gating (Catterall et al. 1992). A series of mutations around the outer vestibule of the channel has recently shown that a tryptophan residue (amino acid number 401) and a glutamine (amino acid number 758) are important determinants of high affinity binding of TTX and STX respectively (Penzotti et al. 1998).

Sodium channels were first purified using their highly specific binding to radiolabelled neurotoxins such as tetrodotoxin or saxitoxin. The purified sodium channel protein was found to consist of various subunits; the α subunit (260kDa) and two different β subunits, β1 (36kDa) and β2 (33kDa) (Messner & Catterall, 1985). The accessory subunits of sodium channels are the β1 and β2 subunits that are important modulators of sodium channel function. Antibodies raised against these accessory subunits immunoprecipitate a channel complex showing an α, β1 and β2 subunit stoichiometry of 1:1:1. The β subunits are not closely related in terms of amino acid sequences but
their secondary structure is similar with each containing a single membrane spanning
segment that separates the extracellular NH$_2$ terminal from the intracellular C terminal
(Isom et al. 1995; Isom et al. 1992). Furthermore, the extracellular domains of the β
subunits both contain motifs of the immunoglobulin superfamily that are similar to
those found in many cell adhesion molecules such as conactin (McCormick et al.
1998; Isom & Catterall, 1996). Although no extracellular ligands have been isolated
this similarity does suggest that the β subunits may play a role in neural cell adhesion
or channel localisation. β$_1$ binds to the α subunit non covalently via hydrophobic
bonds whilst the β$_2$ is covalently linked with disulfide bonds (Catterall, 1992). Both
subunits enhance functional channel expression in Xenopus oocytes (Messner &
Catterall, 1985). The β$_1$ subunit confers faster inactivation kinetics and shifts the
voltage dependence in the hyperpolarising direction, increasing current amplitudes.
The β$_2$ subunit also increases the current amplitude and modulates channel gating
(Isom et al. 1995). Coexpression of β1 and β2 subunits yields sodium channels with
kinetics and voltage dependence that closely resembles native channels and a 5 to 10
told increase of functional channel expression (Isom et al. 1992; Scheinman et al.
1989). The disulfide linkage of β2 subunit to the α subunit is the rate limiting step in
the biosynthesis of sodium channels in the plasma membrane. Most newly synthesised
α subunits are not covalently linked to β2 subunits and remain in an intracellular
metabolically stable pool. During the biosynthesis of α subunits, 30% of them
become covalently linked to the β2 subunit and these complexes are preferentially
targeted to the plasma membrane. The remaining α subunits which were not disulfide
linked to the β2 subunit exist in the cytoplasmic pool. Pulse chase experiments
showed that the α subunits within the intracellular free pool had a half life of 30 hours
whilst the cell surface channels had a half life of 50 hours (Schmidt & Catterall, 1986).

Sodium channels can be modulated by protein kinase C (PKC) or protein kinase A
(PKA) mediated phosphorylation. The intracellular linker regions between domains I
and II contains five consensus sites for cyclic AMP-dependent protein kinase (PKA)
phosphorylation (Kennelly & Krebs, 1991). When oocytes microinjected with mRNA
for rat brain IIA sodium channel were treated with PKA the magnitude of the sodium
current was reduced (Smith & Goldin, 1996). When these phosphorylation sites were
deleted or the serines swapped for alanines by site directed mutagenesis the current reduction resulting from PKA treatment was abolished (Li et al. 1993). Neurotransmitters that are coupled to PKA signaling cascades could therefore have a modulatory effect on sodium currents, and this is the case for the effect of dopamine on sodium currents of hippocampal neurones (Cantrell et al. 1997). In contrast, the inflammatory mediator prostaglandin E\(_2\) (PGE\(_2\)) that is linked to the PKA signaling pathway can increase the TTX-R sodium current magnitude and shift the activation curve in the hyperpolarising direction (England et al. 1996). Phosphorylation can be further regulated at these sites with dephosphorylation by phosphatease 2A (Chen et al. 1995).

PKC phosphorylates a serine residue which is located on the linker regions between domains III and IV (marked as □ in figure 1.8). Phosphorylation at this site also reduces the current magnitude and the rate of fast inactivation (West et al. 1991). It is not surprising that modulation of this region results in alteration of inactivation kinetics as the hydrophobic IMF cluster important for inactivation is also situated on the intracellular linker region connecting domain III and IV.

Since the first sodium channel \(\alpha\) subunit was cloned (Noda et al. 1984) many other genes encoding for sodium channel \(\alpha\) subunits have been characterised. Table 1.3 summarises the properties of the channel types found in the peripheral nervous system in the past decade.
Table 1.3. A table showing the types of sodium channels known to be present in adult DRG neurones. This table was compiled from (Okuse et al. 1997; Black et al. 1996; Sangameswaran et al. 1997) to summarise the diverse range of α sodium channel genes expressed in DRG neurones that have been described over the last decade. Some of the channels types are expressed in the central nervous system (CNS) in addition to the peripheral nervous system (PNS). In the future more α subunit genes will probably be isolated and named using a nomenclature soon to be decided.

The expression of type II sodium channels is regulated at the transcriptional level by a transcription factor REST (RE1 silencing transcription factor). The RE1 silencer element is a 28 bp sequence that confers neuronal specific expression of the type II sodium channel gene. Cell types that contain the transcription factor REST have the type II sodium channel gene expression silenced, whereas neuronal cells that do not contain REST express type II sodium channels (Chong et al. 1995). The molecular mechanisms responsible for regulating differential expression of voltage-gated ion channels in the nervous system are not well understood, but it is possible that REST or other transcription factors with a similar function could control the expression of voltage-gated ion channels.
Voltage-gated ion channels of DRG neurones

DRG neurones, being a heterogeneous population, show a variety of electrical characteristics, such as action potential shape, conduction velocity and firing patterns. The key to the electrical signature of a DRG neurone rests in the nature of the voltage-gated ion channels it contains (Mandel, 1993; Waddell & Lawson, 1990; Yoshimura et al. 1996; Yoshimura & De Groat, 1996). An example of this is the presence of TTX-R sodium channels in small, dark and capsaicin sensitive neurones that fire action potentials with a longer duration than those of neurones with only TTX-S channels (Campbell, 1992; Arbuckle & Docherty, 1995).

Calcium channels have a similar overall architecture as the sodium channels, of four homologous internal domains, but with important differences in regions such as the intra-segment loops which confer ion selectivity. The calcium currents of DRG neurones not only shape the action potential but also act as a second messenger as local concentration of calcium can increase by 4 orders of magnitude around calcium channels (Hille, 1992). Calcium currents can be crudely separated into two subtypes based on their different sensitivity to voltage. The high voltage activated (HVA) currents are activated by voltages greater than -20mV and show very slow inactivation. In contrast, the low voltage activated currents are activated by voltages greater than -50mV and inactivate rapidly.

The potassium channel gene, in contrast to sodium and calcium channel genes, encodes for a protein about a quarter of the size of the sodium α subunit and is equivalent to one domain of the sodium or calcium channels. The pore forming structure is formed when 4 of these subunits come together. The delayed rectifier type potassium currents are responsible for the repolarisation phase of the action potential. There are many different subtypes of delayed rectifier type potassium currents and two can be separated simply in relation to their inactivation properties, one inactivates quickly (~50msec) whilst the other hardly inactivates at all (seconds). These two potassium currents contribute to the action potential shape and subsequently action
potential firing patterns (Villiere & McLachlan, 1996). The inwardly rectifying potassium current has a role in setting the membrane potential and again may contribute to the firing pattern of DRG neurones (Birch et al. 1991). DRG neurones also contain a plethora of ligand gated ion channels in addition to the voltage-gated ion channels.

A summary of the voltage-gated ion currents, other than sodium currents, that were investigated in this study are summarised in the table 1.4 below.
<table>
<thead>
<tr>
<th>Ion channel</th>
<th>Properties</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium HVA</td>
<td>Currents activated by a large depolarisation (~20mV), with slow inactivation kinetics. This current can be further separated pharmacologically.</td>
<td>Fox et al. 1987; Mintz et al. 1992; Hilaire et al. 1996; Kobrinsky et al. 1994.</td>
</tr>
<tr>
<td>L-type</td>
<td>Dihydropyridine (DHP) sensitive.</td>
<td></td>
</tr>
<tr>
<td>N-type</td>
<td>( \omega )-conotoxin GVIA (CgTx) sensitive.</td>
<td></td>
</tr>
<tr>
<td>P-type</td>
<td>DHP and ( \omega )-CgTx resistant but very sensitive (IC(_{50}) 2nM) to ( \omega )-aga-toxin IVA (( \omega )-aga-IVA).</td>
<td></td>
</tr>
<tr>
<td>Q-type</td>
<td>( \omega )-aga-IVA sensitive (IC(_{50}) 140nM).</td>
<td></td>
</tr>
<tr>
<td>Calcium LVA</td>
<td>The T type calcium currents are activated by small depolarisations (~60mV) with fast (~50msec) inactivation kinetics. Studies of the inactivation kinetics have revealed that the LVA currents are composed of heterogeneous currents observed that can be separated pharmacologically based on their sensitivity to the phorbol esters.</td>
<td>Perez Reyes et al. 1998; Bean &amp; McDonough, 1998; Scroggs &amp; Fox, 1992; Feigenspan et al. 1998.</td>
</tr>
<tr>
<td>T-type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium delayed rectifier</td>
<td>Non-inactivating and inactivating type currents that can be separated on the basis of their different inactivation properties. These current types can be separated further biophysically and pharmacologically such as sensitivity to TEA ions or Cs(^+) permeability. The currents are not uniformly expressed in all types of DRG neurones.</td>
<td>Robertson, 1997; Safronov et al. 1996; Gold et al. 1996; Kostyuk et al. 1981.</td>
</tr>
<tr>
<td>Potassium inward rectifier</td>
<td>Currents activated by hyperpolarising steps (between -60mV and -120mV), slowly activating (100msec) and very slowly inactivating (&gt;1sec) kinetics. Currents predominantly found in large diameter DRG neurones.</td>
<td>Mayer &amp; Westbrook, 1983; Scroggs et al. 1994.</td>
</tr>
</tbody>
</table>

Table 1.4. A table summarising the properties of some of the ionic currents found in DRG neurones. The table was compiled from the references listed in the table and from (Nowycky, 1992; Pearce & Duchen, 1994).
The effect of herpes viruses on excitable cells

The effect that herpes virus infections have on the neurones that they infect has been of interest to scientists for many years. *Pseudorabies* virus, which is not a rabies virus as the name suggests but a herpes virus, causes Aujeszky's disease in adult pigs and results in fatal encephalitis in piglets. The virus can form a latent infection in the sensory ganglia as well as undergo a rapid spread through the central nervous system (Hayasaka, 1996). *Pseudorabies* virus had striking electrophysiological effects on sympathetic ganglia infected *in vivo* (Dempsher et al. 1955). Two days after the *Pseudorabies* virus had been inoculated into rat eyes, the cervical ganglion was removed and the cell bodies showed clear histological changes characteristic of viral infection. Electrophysiological experiments, measuring the membrane voltage of these neurones, showed spontaneous and synchronous activity that was never observed in uninfected neurones. Later in the course of infection, the amplitude of the action potential responses was greatly reduced and eventually the neurones became completely unresponsive and unexcitable (Dempsher et al. 1955; Kiraly & Dolivo, 1982).

Nahmias conducted studies investigating the effect of herpes virus infections on many cell types. Fibroblast cells infected with cytomegalovirus and herpes simplex virus developed depolarised resting membrane potentials even to positive values after 12 hours of infection (Fritz, & Nahmias, 1972). Another study investigated chick embryo heart cells. These cells can beat for extended periods in culture and after forming aggregates will beat in a synchronised manner for about 40 minutes (DeHaan & Hirakow, 1972). When these cells were infected with HSV *in vitro* they were very slow to form synchronously beating aggregates. They began to stop beating after 6 hours and after 12 hours they stopped beating altogether (Batra et al. 1976). The action potentials of these infected cardiac cells had a decreased overshoot, a slower rate of rise of the upstroke and were of a longer duration. The infected cardiac cells still excluded trypan blue suggesting that they were still alive during the infection (Shrier et al. 1978). These results demonstrated that *Herpes simplex* virus could alter the electrophysiological properties of excitable cells.
In the early 1980's scientists focused specifically on the effects of *Herpes simplex* virus infection of dorsal root ganglion (DRG) neurones where the virus replicates and forms latent infection *in vivo*. The electrophysiological characteristics of dissociated adult guinea pig DRG neurones infected for 24 hours with HSV-2 *in vitro* were changed. Firstly, many of the neurones were unable to fire an action potential and were unexcitable. Secondly, in neurones that were excitable, the action potentials were characteristically smaller in amplitude and broader in appearance (Fukuda & Kurata, 1981). The DRG neurones of neonatal rats infected with HSV-1 were also reported to have action potentials of a decreased overshoot and increased width at half maximal voltage compared with the action potentials of control, uninfected neurones (Oakes et al. 1981). The maximum rate of rise (Volts/second) of the depolarising phase or upstroke of the action potential was found to be reduced in these infected neurones. Experiments measuring the membrane voltages of infected neurones bathed in solution without sodium ions showed calcium spikes that were comparable with those of control, uninfected neurones. Since the rising phase of the action potential is comprised of sodium and calcium inward currents, these results indicated that the decreased rate of rise of the action potential observed in infected neurones was a function of decreased sodium conductance (Fukuda et al. 1983).

The physiological response of sensory neurones to an infection with HSV-1 was found to be dependent on the strain of virus used (Mayer et al. 1985). There are many different strains of HSV some of which can be differentiated on the basis of their plaque morphology. Cells infected with syncytial strains of HSV show the pathogenic phenomenon where adjacent infected cell membranes fuse to form polykaryocytes or large multi-nucleated cells. Genetic studies have shown that at least 5 genes in the viral genome confer the capacity to fuse cells. Infection of neonatal rat DRG neurones with a syncytial strain of HSV-1 (HSV1, 03syn) for 15-40 hours resulted in spontaneous action potentials in neurones which were usually electrically silent. Pairs of neurones were also noted as having synchronous activity. The neurones did not show signs of forming syncytia although the synchrony of the spontaneous firing suggested that the neurones were probably connected via their neurite extensions. In contrast, infection of DRG neurones with non-syncytial strains of HSV did not result
in spontaneous activity. The spontaneous and synchronous activity described by Mayer was very similar to that observed when sympathetic ganglia were infected with *Pseudorabies* virus (Dempsher et al. 1955). A more detailed analysis of voltage activated currents during an infection with different strains of HSV showed that an infection with non syncytial strains resulted in the loss of hyperpolarisation activated potassium currents and a loss of excitability whilst the outward potassium conductances remained unchanged. DRG neurones infected with syncytial strains (HSV1, 03syn), however, remained excitable and in some cases were spontaneously active and the hyperpolarisation activated currents were also unchanged (Mayer et al. 1986; Mayer, 1986). This illustrates that the electrical changes that were induced by HSV infection in neurones were dependent on the strain of virus.

In summary, neurones infected with HSV showed different excitable properties such as loss of excitability, change in action potential shape, possibly due to loss of sodium conductance, whilst the calcium currents remained unchanged. Infection with syncytial strains of HSV resulted in synchronous, spontaneous activity, whereas an infection with non-syncytial strains resulted in loss of the hyperpolarisation activated currents and loss of excitability.

The improvements in the patch-clamp technique provide experimenters with the ability to make high resolution ionic current recordings (Hamill et al. 1981). The first goal of this study was to use the patch-clamp technique in the voltage-clamp mode to determine the specific current changes that underlie the previously reported alteration in action potential shape. Although HSV has been extensively characterised there have been no comments in the literature about which aspects of viral infection may give rise to the changes in excitability in infected DRG neurones. Once having discovered the specific ionic current changes and the aspects of viral infection involved in these changes the next aim was to investigate the cellular mechanisms governing this process. Developments in gene therapy vector design have resulted in HSV based vectors that can introduce foreign genes into neurones. The effect these viral vectors have on the electrophysiological properties of neurones is unknown. The
patch-clamp technique was used to investigate the electrophysiological side effects of gene therapy with HSV based viral vectors in DRG neurones.
Chapter 2

Materials and Methods

This chapter describes the methods used to obtain and analyse the results in chapters 3-6. The aim of these experiments was to investigate the effects of wt HSV 17^ infection on the electrophysiological characteristics of adult rat DRG neurones. Electrophysiological recordings of DRG neurones were made using the patch-clamp technique in the whole cell configuration to either measure membrane voltage or whole cell currents. The DRG neurones were infected with wt HSV 17^ or treated with various biochemical agents for experiments designed to determine the mechanisms underlying the effects of wt HSV 17^ infection in these neurones. The rest of the chapter describes firstly, the preparation of neurone cultures, viral stocks, and visualisation of infected neurones. Secondly, there is a description of the solutions and voltage protocols used to make electrophysiological recordings and the methods used to analyse the results. Finally, this chapter explains the techniques used to measure protein synthesis, western blot analysis and cosmid DNA preparation.
Preparation of Dorsal Root Ganglion (DRG) Neurones

DRG neurones were prepared from adult (~200g) male or female Sprague-Dawley rats (CD rats Charles River) as described previously (Bevan & Winter, 1995). In brief, adult rats were killed by CO₂ asphyxiation. Spinal ganglia were removed aseptically from all levels of the spinal cord. Ganglia were incubated for 3 hours in 0.125% collagenase (Worthington, Type IV) in F-14 medium (Imperial) with 2mM L-Glutamine and 10 μg.ml⁻¹ penicillin, at 37°C in a humidified incubator gassed with 3% CO₂ in air. Cells were dissociated by trituration through a flame polished Pasteur pipette. The cells were centrifuged through 2mls of 15% bovine serum albumin in F-14 medium and the pellet was resuspended in F-14 with 4% Ultroser G (Gibco), 2mM L-Glutamine and 10 μg.ml⁻¹ penicillin and streptomycin and 50ng.ml⁻¹ nerve growth factor (Promega).

For culture, the cells were plated onto sterile 13mm glass coverslips (BDH) previously coated with 10μg.ml⁻¹ poly-D-ornithine and 5μg.ml⁻¹ laminin (Gibco) and maintained overnight at 37°C in a humidified incubator gassed with 3% CO₂ in air. For replating, the cells were first plated onto 35mm petri dishes (Nunc) previously coated with poly-D-ornithine and laminin, at a density of about 1-2x10⁶ cells/plate. When the cells were needed for electrophysiological study they were aspirated from the petri dish and plated onto poly-D-ornithine and laminin coated 13mm coverslips. Cells were studied either 4-12 hours after dissociation or 2-12 hours after replating. The replating step ensured that the cells were essentially spherical and without obvious neurites which improved the integrity of the voltage clamp. Electrophysiological recordings were made from DRG neurones that ranged from 10μm to 50μm in diameter which spanned the size distribution of all neurones in the culture.

For experiments with cycloheximide, DRG neurones were incubated with 50 μg.ml⁻¹ cycloheximide in F-14 medium for 24 hours. For cells that were treated with cycloheximide and HSV-1, the cells were incubated with cycloheximide for 1/2 hour before the infection with HSV-1 in order to disrupt the cell’s existing protein synthesis. Other biochemical agents used were 50 μM Acyclovir (Calbiochem), 1μM bafilomycin A₁, 100μM chloroquine and 25mM ammonium chloride, 0.1 mM Veratridine and 5mM TTX (Calbiochem).
All experiments were performed at room temperature, typically 22°C, unless otherwise stated. All chemicals were supplied by Sigma unless otherwise stated.

**Preparation of Viral Stocks**

Wild type virus HSV-1 Glasgow strain 17 syn⁺ (wt HSV 17⁺) (Brown et al. 1973) was used in this study. HSV-1 was routinely propagated in BHK C 13 cells (baby hamster kidney cell line) (MacPhearson & Stoker, 1962) which were grown in DMEM (Dulbecco’s Modified Eagle’s Medium) (Gibco) with 10% fetal calf serum (Gibco) and 1% penicillin and streptomycin (Gibco). wt HSV 17⁺ was collected from the culture after a series of rapid freeze thaw cycles and was titrated on Vero cells (an African green monkey kidney cell line) grown in RPMI 1640 (Gibco) with 10% Fetal calf serum, 1% penicillin and streptomycin and 2% sodium bicarbonate (Gibco).

HSV-1 deletion mutant of ICP4 gene (D30EBA) (Paterson & Everett, 1990) was propagated and titrated on E26 cells (Samaniego et al. 1995), a Vero derived cell line that expresses complementing levels of wild type ICP4, grown in RPMI 1640 with 10% Fetal calf serum, 1% penicillin and streptomycin, 2% sodium bicarbonate and 500µg.ml⁻¹ geneticin (G418 Gibco).

HSV-1 deletion mutant of ICP27 gene (ZAMN⁺) (Howard et al. 1997) was propagated and titrated in B130/2 cells, a BHK derived complementing cell line expressing wild type levels of ICP27 upon infection, grown in DMEM (Dulbecco’s Modified Eagle’s Medium) with 10% fetal calf serum, 1% penicillin and streptomycin and 500µg.ml⁻¹ geneticin. Viruses were always seeded in the absence of G418. All the cell cultures were maintained at 37°C at 5% CO₂, in a humidified incubator.

The virus stocks were titrated by infecting confluent Vero cell monolayers with serially diluted virus. The cultures were incubated for 1 hour in serum free media (RPMI 1640) to allow viral attachment and penetration. These cultures were then incubated with media containing serum under a layer of 0.5% carboxy-methyl cellulose (CMC) overnight. This method was used so that one virus would cause one plaque and was unable to spread through the CMC and produce another plaque. The cells were then fixed with a solution of 3:1 methanol:actetic acid and the cells were
visualised by staining with crystal violet. The plaques were counted and the p.f.u/ml of the stock solution was calculated, usually about $5 \times 10^8$ p.f.u/ml.

**Immunohistochemistry**

DRG neurone cultures were grown as described above and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 20 minutes. The coverslips were washed in PBS, air dried and mounted with the cells uppermost on microscope slides for easy handling. The primary antibodies used were a murine monoclonal anti ICP4 antibody (Autogen Bioclear, UK, Ltd.). The antibody was diluted 1 in 200 in PBS with 10% fetal calf serum (FCS) and 0.1% Triton X-100 and 0.02% sodium azide, added to the samples and incubated overnight at 4°C. The samples were then washed in PBS and 0.01% Triton X-100 and then incubated for 1 hr at room temperature with a biotinylated anti-mouse antibody (Jackson Immunoresearch, USA.) diluted 1 in 200 in PBS with 10% fetal calf serum (FCS) and 0.1% Triton X-100 and 0.02% sodium azide. The samples were then washed in PBS and 0.01% triton X-100 and incubated for 1 hr at room temperature with streptavidin-fluorescein diluted 1 in 100 in PBS with 10% fetal calf serum (FCS), 0.1% Triton X-100 and 0.02% sodium azide. Finally, the slides were washed in PBS and 0.01% triton X-100 again and mounted in Citifluor (UKC, UK) and visualised under a fluorescence objective (excitation 470-490nM) of a brightfield (Nikon Fluophot) microscope.

Indirect immunohistochemistry was used to determine the proportions of DRG neurones that were infected 4 hours and 24 hours post infection. To ensure that replication of HSV-1 in non-neuronal cells with secondary infection of DRG neurones was not occurring, immunohistochemistry was performed at 4 hours post infection. These studies showed that all the neurones were infected at the time of inoculation with 5 pfu of wt HSV 17 virus per DRG neurone.
**β-Galactosidase staining**

Culture medium was removed from the DRG neurones after 24 hours of infection and the cultures fixed in 0.05% glutaraldehyde at room temperature for 15 minutes. The cultures were then washed twice in phosphate-buffered saline (PBS) and then stained with X-gal (Melford Laboratories, Ipswich, UK) for 1 hour at 37°C in 5 mM potassium ferrocyanite, 5 mM potassium ferricyanide, 2 mM magnesium chloride, 1 mg ml⁻¹ X-gal, 2.4% dimethyl sulfoxide DMSO in PBS.

**Electrophysiological recordings**

The electrophysiological recordings made in these experiments used the patch-clamp technique in the whole cell configuration. It is possible either to clamp the current and measure membrane voltage or clamp the voltage and measure membrane currents. In the current-clamp mode the membrane voltage was recorded in response to injections of current, this mode was used to generate action potentials. In the voltage-clamp mode the membrane voltage of the neurone was controlled and the whole cell currents were evoked by changes in voltage.

The voltage-clamp technique was developed by Andrew Huxley and Alan Hodgkin (1952) based on the original ideas of Marmont (1949) for investigating the changes in permeability which gave rise to the action potential in the squid axon. The usefulness of the voltage clamp technique is that it allows the separation of the capacitative currents associated with charging up the cell membrane during voltage changes, from the ionic currents through voltage-gated ion channels. The first series of voltage-clamp experiments using the “patch clamp” whole cell methods had low resistance seals (~50 MΩ) between the pipette and the membrane and the resolution of the recordings was poor. The voltage-clamp technique was greatly improved when it was discovered that slight suction could induce very tight seals with an order of magnitude higher resistance. This event is now known as gigaseal formation (Hamill *et al.* 1981).
The gigaseal formation allowed for much higher resolution recordings, even of single channel currents, due to the reduced leakage current around the seal. These very tight, mechanically stable membrane seals could be formed with a resistance of giga-ohms \((10^9 \Omega)\), gigaseals and could be manipulated in a number of ways in order to record currents either from the whole cell or just from a patch of membrane.

To form the high resistance seal the pipette was manipulated so that it was close to the neurone and the resistance was monitored on the oscilloscope by applying a small voltage step of about 5mV. The pipette was placed lightly on the plasma membrane of the neurone and the very tight seal was formed with slight suction to the inside of the pipette. Further suction or a very brief 1V voltage pulse was applied to break the membrane under the tip of the pipette. This rupture of the membrane patch gave continuity between the pipette and the whole cell contents. A large capacity transient in response to an imposed voltage step of the cell membrane appears on the oscilloscope when a whole cell configuration has been successfully achieved. The importance of the giga seal stems from the fact that, when the seal resistance is high enough, essentially all of the current passes through the comparatively low access resistance from the pipette to the cell interior. When the pipette seal is very high there is no current leak between the pipette and the cell membrane such that high resolution, low noise recordings can be made (Neher, 1992).

To enhance the success of gigaseal formation care was taken to reduce debris and any dirt that could interfere with the process of seal formation. Solutions were filtered (0.2\( \mu \)m filter Sartorius) to remove dust and positive pressure was applied to the pipette as it was drawn towards the neurone in order to "blow away" any cell debris from the pipette tip. The pipette tips were fire polished in a heated microforge under the orange heat of the platinum wire. This process smoothed the tip and prevented piercing of the neuronal membrane, aiding the seal formation process. The temperature at which the borosilicate glass used for the patch pipettes softens is between 700-800°C which means that it can be easily tapered into a shape of appropriate width and bluntness for gigaseal creation (Ogden & Stansfield, 1994). The composition of the glass pipette also influences its ability to seal to neuronal membranes. Some types of glass will
leach chemicals such as arsenic or rubidium that are known channel blockers which could interfere with ion channel current recordings. The borosilicate glass used for these experiments is not known to leach any substances. The pipettes contained a fused fibre that allows the internal solution to be back filled. Stainless steel needles were not used for backfilling in case of leaching of heavy metal ions and nonmetallic syringe needles were used instead (World Precision Instruments, Inc.).
A flow chart summarising the electrophysiological set up is shown in figure 2.1.

Figure 2.1. A schematic representation of the electrophysiological set up for patch-clamp recording in the whole cell mode. DRG neurones were voltage-clamped in the whole cell configuration with an Axopatch 200A amplifier (Axon Instruments). Voltage-clamp potentials were generated by a IBM compatible personal computer (Dell 486) and applied through an analogue-digital interface (DMA TL-1) using PCLAMP software (Axon Instruments). Borosilicate patch pipettes were pulled from 1.5 mm external diameter glass fibre-filled capillaries (Clark Electromedical GC150-F10) in a three stage pull on a P-80 Brown-Flaming micropipette puller (Sutter Instrument Co.). The pipettes were fire polished and had a final resistance of 2-5 MΩ when filled. Pipette and membrane capacitance was compensated using the circuitry on the amplifier and series resistance compensation (~80%) was employed.
There are many sources of error that can occur whilst recording in the whole cell mode that can be compensated for in a number of ways. The first of these errors revolves around the fact that the plasma membrane act like a capacitor with an intrinsic capacitance of 1µF.cm⁻². If the membrane voltage is stepped to a new voltage, then a current is required to charge the membrane capacitance and this slows the speed for the membrane to reach the new potential. Capacitance compensation using the patch-clamp amplifier ameliorates this error by injecting a current that cancels the capacitative currents. Another source of capacitative error arises from the glass pipettes used. The glass forms an insulating gap between the internal and external solutions forming a capacitor. Since capacitance is inversely proportional to the distance between the conducting sheets, increasing the thickness of the pipette glass by using electrodes with thicker walls had the effect of reducing the stray capacitance. The properties of the glass pipette contribute to the level of capacitance errors and the type of glass was chosen in order to reduce the contribution to capacitative error. Borosilicate has the smallest slow capacity transients when it charges, with less noise compared to other types of glass and was the natural choice (Rae & Levis, 1992). Further reduction of pipette/bath stray capacitance can be achieved by coating the patch pipettes with Sylgard, a resin that thickens the walls of the pipettes, but for these experiments the recording resolution was high enough without the need for Sylgard coating.

Another source of error arises from the resistance of the pipette in series with the cell. This resistance is called the series resistance. It originates from many factors such as the glass pipettes and cytoplasmic resistance (or access resistance), and for these experiments was typically between 2-5MΩ. In order to compensate for the potential "lost" across the series resistance, the amplifier was set to increase the command potential such that the membrane experiences a potential closer to that intended. This is known as series resistance compensation (see e.g. Halliwell et al. 1994).
Solutions and Voltage Protocols

The solutions used to isolate specific ionic currents are shown in the tables below. The voltage protocols used to evoke various voltage-gated ion currents are also shown below. The numerical values in the figures showing the voltage protocols indicate the voltage in mV.

"Physiological" solutions

<table>
<thead>
<tr>
<th>External solution</th>
<th>Internal solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>130</td>
</tr>
<tr>
<td>KCl</td>
<td>3.5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40</td>
</tr>
<tr>
<td>pH 7.4 titrated with NaOH</td>
<td>pH 7.2 titrated with KOH</td>
</tr>
</tbody>
</table>

These solutions were designed to resemble the intracellular and extracellular compositions found physiologically in DRG neurones and were used to measure changes in membrane voltage under current-clamp (Pearce & Duchen, 1994). Action potentials were evoked by current injections of several pA.
Solutions for sodium current isolation

<table>
<thead>
<tr>
<th>External solution</th>
<th>Internal solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>50</td>
</tr>
<tr>
<td>Choline.Cl</td>
<td>100</td>
</tr>
<tr>
<td>TEA.Cl</td>
<td>20</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>40</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10μM</td>
</tr>
<tr>
<td>pH 7.4 titrated with KOH</td>
<td></td>
</tr>
</tbody>
</table>

Sodium currents were isolated by blocking potassium channels with cesium as the major internal cation and with tetraethylammonium (TEA) ions present externally. The calcium currents were blocked with 10μM calcium in the external solution. This small amount of calcium was sufficient to bind to high affinity binding sites within the calcium channel and prevent them from becoming sodium conducting but did not generate a measurable calcium current (Kostyuk & Krishtal, 1977).

A series of different sodium current traces were evoked by the voltage protocols described below (Elliott & Elliott, 1993). A single sodium current trace was evoked by a voltage step from the holding potential (-80mV) to a command potential of +10mV.

![Voltage protocol](image)

**Figure 2.2. The one step depolarisation protocol.** A 13 msec step depolarisation from the holding potential -80mV to +10 mV, was used to generate a single sodium current.
The TTX-S and TTX-R sodium currents were separated electrophysiologically on the basis of their different inactivation properties. The total sodium current was evoked by a 10 msec depolarising command step to -20 mV from a prepulse potential of -120mV. TTX-R sodium current was evoked from a prepulse potential of -50mV, as shown in figure 2.3. The TTX-S sodium current was obtained by subtracting the TTX-R current from the total sodium current.

![Figure 2.3](image)

**Figure 2.3. The voltage protocol for separating the TTX-S and TTX-R components of the sodium currents.** First, the total sodium current was evoked after a prepulse to -120mV by a depolarising 10 msec step to -20mV. Second, the TTX-R component of the sodium current was evoked after a prepulse to -50mV by another step to -20mV. Third, the TTX-S component could be separated by subtracting the TTX-R current trace from the total current trace.

A family of all the sodium currents was evoked after a hyperpolarising prepulse to remove inactivation by a series of depolarising 5mV steps from -50mV to +25mV as shown in figure 2.4.

![Figure 2.4](image)

**Figure 2.4. The voltage protocol used to evoke a family of sodium currents.** A family of sodium currents was generated after a prepulse to -120mV to remove sodium channel inactivation, using 10 msec, 5mV step depolarisations from -50mV to +25mV.
The steady-state inactivation was investigated with the voltage protocol, shown in figure 2.5, with prepulse potentials of 230 msec durations to various potentials between -120mV and -5mV and a test potential step to 0mV.

![Figure 2.5. The voltage protocol used to investigate the steady state inactivation of sodium currents.](image)

Prepulses of 230 msec to potentials ranging from -120mV to -5mV, were followed by a 10 msec command potential to 0mV.

**Solutions for calcium channel current isolation**

<table>
<thead>
<tr>
<th>External solution</th>
<th>Internal solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical</td>
<td>mM</td>
</tr>
<tr>
<td>TEA Cl</td>
<td>120</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
</tr>
<tr>
<td>CaCl2/BaCl2</td>
<td>5</td>
</tr>
<tr>
<td>EGTA</td>
<td>pH 7.2 titrated with TEA-OH</td>
</tr>
</tbody>
</table>

In most experiments barium was used as a charge carrier to allow longer recordings without inactivation of the channels: ATP and GTP were added to in the internal solution to delay the onset of rundown (Bean, 1992). The voltage protocols used to investigate the characteristics of calcium channel currents are shown in figure 2.6.
Figure 2.6. A diagram showing the different protocols used to evoke calcium channel currents. a The low voltage activated (LVA) calcium channel currents were evoked by a depolarising step to -40mV that inactivated the LVA calcium channels such that the only the high voltage activated (HVA) calcium channel currents were evoked by a second depolarising step to 0mV. b A family of calcium channel currents were evoked by a series of command potentials ranging from -80mV to +40mV (360 msec). c The inactivating components of the calcium channel currents were investigated with a protocol that elicited currents with a 320msec command potential to -10mV, after a prepulse that ranged from -100mV to +15 mV.

Solutions for delayed rectifier type potassium current isolation

<table>
<thead>
<tr>
<th>External solution</th>
<th>Chemical</th>
<th>mM</th>
<th>Internal solution</th>
<th>Chemical</th>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>choline</td>
<td>130</td>
<td></td>
<td>KCl</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td></td>
<td>KF</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>10</td>
<td></td>
<td>EGTA</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.2</td>
<td></td>
<td>MgCl₂</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>3</td>
<td></td>
<td>HEPES</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.2 titrated with K-OH</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.4 titrated with KOH</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Several voltage protocols were used to evoke delayed rectifier type potassium currents to investigate the different electrophysiological characteristics of these currents (Gold et al. 1996) and are illustrated in figure 2.7.
Figure 2.7. **The voltage protocols for evoking delayed rectifier type potassium currents.**

a Total outward potassium currents were generated with a 40 msec depolarising step to +10mV after a prepulse to -80mV. After a prepulse to -50mV the non-inactivating type outward potassium current was evoked from a 40 msec test potential of +10mV. b A family of delayed rectifier type potassium currents was evoked by a series of 15 msec depolarising command potentials in the range of -90mV to +40mV. After each depolarisation the voltage was stepped back to -40mV. c Currents were evoked by a step to +45mV after a series of prepulses ranging from -120mV to 0mV in order to investigate the steady-state inactivation of the inactivating component of the potassium currents.

### Solutions for inward rectifier type potassium current isolation

<table>
<thead>
<tr>
<th><strong>External solution</strong></th>
<th><strong>Internal solution</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical</strong></td>
<td><strong>mM</strong></td>
</tr>
<tr>
<td>NaCl</td>
<td>140</td>
</tr>
<tr>
<td>KCl</td>
<td>4</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td>glucose</td>
<td>10</td>
</tr>
<tr>
<td>pH 7.4 titrated with NaOH</td>
<td></td>
</tr>
</tbody>
</table>

Different solutions were used for the isolation of the inwardly rectifying potassium currents. This is because the channels were also permeable to sodium ions and rectification is conferred by magnesium ions (Scroggs et al. 1994). The voltage protocols used to generate inwardly rectifying potassium currents are displayed below.
In addition to voltage clamp recordings the time dependent rectification of membrane voltage was also recorded in the current clamp mode with series of current injections.

**Figure 2.8. The voltage protocols used to evoke inwardly rectifying type potassium currents.**  
**a** The voltage protocol used to generate a single inwardly rectifying potassium current shown is a hyperpolarising step from -70mV to -120mV for 900 msec.  
**b** The voltage protocol employed to evoke a family of inwardly rectifying potassium currents was a series of hyperpolarising steps from -50mV to -140mV for 1 sec.

**Junction Potentials**

When two solutions of different compositions come into contact the different mobilities of the anions and cations may result in the formation of a junction potential \( V_{LJ} \) at the liquid interface. In these experiments a junction potential will occur between the bath (external) solution and the pipette (internal) solution leading to erroneous recordings of membrane potential. The liquid junction potential is typically 2-12mV and can not be eliminated by zeroing the patch clamp amplifier prior to seal formation (Barry & Lynch, 1991). When the amplifier was zeroed prior to the pipette contacting the neurone any existing junction potential was canceled with an equal and opposite correction potential within the amplifier. When the seal was broken, as for recording in the whole cell mode, the pipette solution initially forms an interface with the contents of the neurone. However, a new junction potential is assumed not to occur at this interface as the pipette solution diffuses into the cell and fully dialyses the cell contents. The problem arises because the correction potential applied to the liquid junction potential between the external and internal solutions remains. For example, if the correction potential is 12mV, then there will be a corresponding error in output voltage. The way to solve this problem is to measure the potential of the bath solution.
with respect to the pipette solution and simply subtract it from the output voltage as shown in equation (1) below (Neher, 1992).

\[ V_m = V - V_{LJ} \]  

Where \( V_m \) is the membrane potential, \( V \) is the voltage output reading on the patch clamp amplifier and \( V_{LJ} \) is the junction potential. For these experiments the junction potential was measured by filling the pipette with internal solution and placing it in a bath also containing internal solution, and then the amplifier was zeroed. The solution in the bath was then switched to external solution and the voltage output on the patch clamp amplifier was recorded. (It should be noted that the polarity of the voltage is opposite to that required for the equation. This is because convention states that the junction potential is the bath solution with respect to the pipette solution.) The junction can also be calculated from the Henderson liquid junction potential equation. The junction potentials of the solution used in the study are shown in table 2.1. The junction potentials were compensated for when the value was above 4mV as the errors involved in recording stable junction potentials did not allow for more accurate measurements.

<table>
<thead>
<tr>
<th>Solution (external)</th>
<th>Liquid junction potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological</td>
<td>+7.3 mV</td>
</tr>
<tr>
<td>Sodium current</td>
<td>+1.1 mV</td>
</tr>
<tr>
<td>Calcium current</td>
<td>+6.1 mV</td>
</tr>
<tr>
<td>Potassium (delayed rectifier) current</td>
<td>+1.2 mV</td>
</tr>
<tr>
<td>Potassium (inward rectifier) current</td>
<td>+3.4 mV</td>
</tr>
</tbody>
</table>

Table 2.1. The junction potentials of the electrophysiological solutions used in this study. The junction potentials is the voltage of the external solution with respect to the internal solution. The values shown in the table are the values used in the equation \( V_m = V - V_{LJ} \) and are of the opposite polarity to the actual values measured.
Analysis of Macroscopic Currents

The hallmark of the voltage gated ion channels is their selective ion conductance evoked, on a millisecond time scale, by a change in membrane potential. The current-voltage relationship of a specific ion channel is determined by the effect of membrane potential, open probability and driving force on the permeable ions. Plotting the maximum current amplitude against the voltage step used to evoke the current gives a characteristic curve for the ion carrying the current through a specific channel. The rate at which ions move through ion channels is determined by a number of factors such as, the magnitude of the electrochemical gradient and membrane potential.

The current-voltage relationship is a summary of the voltage dependence of an ion channel. The shape of the current-voltage relationship for the current through these channels is determined by the channel open probabilities and the driving force on the permeant ions. For any given ion (X^+) the zero current or reversal potential (E_{rev}) occurs at the potential where the concentration gradient is balanced by the electrical gradient across the membrane and can be calculated from the Nernst equation (2):

\[
E_{rev} = \frac{RT}{zF} \ln \left( \frac{[X]_o}{[X]_i} \right)
\]  

(2)

Where R is the gas constant (8.314 JK^{-1} mol^{-1}), T is the absolute temperature (K), z is the charge number for the ion, F is the Faraday constant (96 500 C mol^{-1}) and [X]_o and [X]_i are the concentrations of X ions on the outside and inside of the membrane. Thus the reversal or zero current potential determined from a current-voltage relationship, gives information about the ion that may be carrying the current. Thus, the driving voltage the ion experiences can be better described as E- E_{rev} (where E is the membrane potential). The conductance, g, of an ion channel is the reciprocal of its resistance, R and can be defined by equation (3):

\[
R = \frac{1}{g}
\]  

(3)
From Ohm’s law, equation 3 can be rewritten in terms of conductance (g) and voltage (E-E_{rev}), such that the equation now becomes equation (4):

\[ g = \frac{I}{E - E_{rev}} \]  

(4)

Where I is the membrane current. Plotting the conductance against the test potential gives the conductance plot or activation curve which is used to characterise voltage-gated ion channels. Conductance changes with voltage depending on the voltage dependence of the open probability of the channel. This curve relationship, can best be described by a Boltzmann function that gives a sigmoid curve of the form (5):

\[ g = \frac{1}{g_{max} \left(1 + \exp \left(\frac{(V_{50} - V)}{k}\right)\right)} \]  

(5)

Where g is conductance, V_{50} is the potential where the conductance is half maximal, V is the test potential and k is the slope factor. The slope factor can provide an estimate of the charge movement during channel gating and indicates the voltage-dependence of the channel activation. The steeper the slope, the greater the voltage-dependence. The slope factor value is the voltage required for a e fold increase in gating charge movement per kT/e. Where k is the Boltzmann’s constant, T is temperature, e is the elementary charge and z is the gating charge valence. The Boltzmann constant, temperature and elementary charge are constant values, thus the gating charge, z, for opening a channel can be calculated (Hille, 1992).

The steady-state inactivation, availability parameter (h_{oo}) was achieved by normalising the current evoked after a prepulse potential (I_{pp}) to the maximum current recorded (I_{max}). The h_{oo} value (I_{pp}/I_{max}) was plotted against the prepulse potential and fitted with the equation (6):

\[ h_{oo} = \frac{1}{1 + \exp \left(\frac{(V - V_{50})}{k}\right)} \]  

(6)
Where $V$ is the prepulse potential, $V_{50}$ is the prepulse potential at which $h^\alpha$ is half maximal and $k$ is the slope factor (Huxley & Hodgkin, 1952; Elliott & Elliott, 1993).

**Statistical Analysis**

Results are presented as mean ± standard error of the mean (s.e.m). In order to investigate the probability that the results of an experiment occurred by chance, one of three statistical tests were performed depending upon the experimental conditions. For experiments that had two experimental conditions with normally distributed results, the Student’s t test was performed. For experiments with more than two experimental conditions, with normally distributed results, the results were statistically tested with Tukey’s post hoc analysis, after analysis of variance. This analysis first considered the amount of variance due to the different treatments, and then compares results from each treatment with error variance due to unknown factors within these experimental conditions. The post hoc analysis calculated the probability that any difference between results from several experimental conditions was due to chance fluctuations. In some cases, however, the distribution of the data was not normal. For example, the sodium current amplitudes of wt HSV 17+ infected DRG neurones were not normally distributed, since a population of the neurones tested showed no measurable sodium current. In these situations a non-parametric statistical analysis, the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison was performed. Non-parametric tests rank-order data and then calculate whether the distribution of high or low ranks is due to experimental conditions or chance. The statistical package used to analyse statistical significance was Systat for Windows, version 7.01.
**Assay for Protein Synthesis**

To examine the effect of cycloheximide on the protein synthesis, DRG neurone cultures were treated with 180μM (50μg.ml⁻¹) cycloheximide and incubated for a further half an hour to allow time for ongoing protein synthesis to finish. The DRG neurone cultures were then incubated in methionine free F14 medium that was supplemented with radiolabeled [¹³⁵S] methionine (15-25μCi.ml⁻¹) and incubated for 24 hours at 37°C. The cultures were washed twice in methionine free medium, the neurones were scraped off the 35mm wells and suspended in medium. Bovine serum albumin (BSA) was added to the suspension as a carrier protein, then 20% trichloroacetic acid (TCA) was added to precipitate the proteins. Samples were then incubated on ice for 4 hours and the precipitate was collected by filtration. GF/F filters (Whatman) wet with a solution of 5% TCA in 0.2% KCl were used, and any excess TCA was washed away with 70% ethanol to avoid quenching. The filters were placed in scintillation vials with scintillant (Beckman) and counted on a Wallac 1409 liquid scintillation counter.

**Western Immunoblot Analysis**

DRG neurones were incubated in F14 medium and pulsed at 2 day intervals with 1-β-D-Arabinofuranosylcytosine (Calbiochem) in order to kill the dividing cells and enrich the culture with neurones. After 2 weeks half of the pure DRG neurone cultures were infected with wt HSV 171 and DRG neurones were harvested in a lysis buffer (10mM Tris-HCl pH7.6, 5mM EDTA, 50mM NaCl, 30mM Na₄P₂O₇.10H₂O, 50mM NaF, 100mM Na₃VO₄, 1% Triton X-100, 1mM PMSF). Protein concentration was assayed using the Bradford method (Biorad). 30μg protein was added to Laemmli buffer (2% SDS, 10% glycerol, 100mM DTT, 60mM Tris pH6.8, 0.001% Bromophenol Blue), incubated at 37°C for 15 minutes and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Separated polypeptides were electrophoretically transferred to nitrocellulose filters (Scheicher and Schuell) at 30V
overnight. The filters were rinsed with TBST buffer (20 mM Tris base, 137 mM sodium chloride, 1 mM hydrochloric acid, 0.1% Tween-20), blocked in 5% milk proteins (Marvel) in TBST for 30 minutes and then treated for 30 minutes with 1 μg.ml⁻¹ anti-Na⁺ channel antibody (Upstate Biotechnology Incorporated) directed against the highly conserved region in the intracellular loop II-IV which is identical in sodium channel subtypes of all known vertebrates. After 3-5 rinses with TBST the filters were incubated with an anti rabbit HRP (horse radish peroxidase) conjugated antibody (Amersham) diluted 1 in 10000 in 1% milk proteins (Marvel) in TBST. The filters were then rinsed in TBST and the proteins visualised using the enhanced chemiluminescence (ECL) western blot kit (Amersham).

**Cosmid Preparation**

DNA manipulations including restriction endonuclease digestions and bulk cosmid preparations were carried out by standard methods (Sambrook *et al.* 1989). The cosmid library consisted of five cosmids containing large wt HSV 17⁺ DNA fragments, which overlap and comprise the entire wt HSV 17⁺ genome (Cunningham & Davison, 1993). This is represented schematically in figure 2.9.

**Figure 2.9.** A schematic diagram showing the locations of cosmids 6, 14, 28, 48 and 56 with respect to the HSV genome. The two unique regions (U), long (L) and short (S) are shown by the single line that are flanked by the inverted repeats (rectangles). The locations of the cosmids is indicated by the open bars. This figure has been adapted from (Cunningham & Davison, 1993).
A cosmid is a combination of a \( \lambda \) based vector and a bacterial plasmid. A \( \lambda \) based vector is derived from a bacteriophage that has a head that is packaged with DNA and a tail for attachment to the bacteria. The phage DNA contains a recognition site, called a \textit{cos} site, which is necessary for packaging of DNA into the head of the phage. A cosmid is simply a large plasmid with \textit{cos} sites. The cosmid genome contains a selectable marker, such as an ampicillin resistance gene. This is so that when the phage infects bacteria, colonies will be formed on selective media. The attractive feature of a cosmid is that it can contain DNA inserts up to 52kb. The five cosmids consisting together of the entire wt HSV 17\textsuperscript{th} genome were supplied as agar cultures of \textit{Escherichia coli} NM554 containing cosmid DNA (Cunningham & Davison, 1993).

Maxi-preparations of cosmids were made by alkaline lysis (Sambrook \textit{et al.} 1989; Birnboim & Doly, 1979). For extraction of cosmid DNA, the bacterial cells were grown overnight in Luria broth (LB) with 100 \( \mu \text{g.ml}^{-1} \) ampicillin (Gibco). The bacterial cells were harvested by centrifugation at 4000g for 10 minutes at 4\( ^\circ \text{C} \). The cells were washed in ice cold STE (0.1 M NaCl, 10 mM Tris at pH 8, and 1 mM EDTA). The bacterial cell pellet was resuspended in 10 ml of solution I (50 mM Tris at pH 8, and 10 mM EDTA). 20 ml of solution II (0.2 N NaOH and 1% SDS) was then added to the solution I and cell mixture and gently mixed before being left on ice for 5 minutes. Following this 15 ml of ice cold 5 M potassium acetate (pH4.8) was then added and again the solution was left on ice for 15 minutes. This mixture was then centrifuged at 3000g for 15 minutes at 4\( ^\circ \text{C} \). The supernatant was poured through gauze to retain the debris and chromosomal DNA aggregates. The supernatant was mixed with 0.6 volumes of isopropanol and left at room temperature for 15 minutes. The DNA precipitate was collected by a 30 minute centrifugation at room temperature at 10000g. The pellet was washed in 70% ethanol and the pellet was, when dried, suspended in 8 ml of TE (1 M Tris at pH 8 and 0.5 M EDTA at pH 8). The cosmid DNA was then ready to be purified by cesium chloride (CsCl) density gradients which was achieved as follows: For every millilitre of cosmid DNA, 1 g of CsCl was added, followed by 50 \( \mu \text{l} \) of ethidium bromide. This solution was placed in ultracentrifuge tubes (Beckman) for centrifugation at 45 000 rpm overnight at 20\( ^\circ \text{C} \) (Sorvall vti 65 rotor). The cosmid DNA could be visualised under u.v. light showing
the lowest band which was removed from the tubes with a hypodermic syringe. The ethidium bromide was removed by first adding TE to the cosmid DNA and an equal volume of isoamyl alcohol. When the solution had settled the lower aqueous phase was transferred to a new vial. This procedure was repeated until the pink colour of the ethidium bromide was no longer visible in the aqueous layer. This aqueous phase was then dialysed against TE overnight. A 1/10 volume of 3 M sodium acetate (pH 5.6) along with 2 volumes of ethanol, was added to the contents of the dialysis bag (Spectrum) and left at -20°C overnight. This was then centrifuged at 9000 rpm for 30 minutes. The pellet was washed with 70% ethanol, air dried and resuspended in 150μl of water ready for microinjection.

In order to check that the cosmid DNA was correct, restriction digests were performed on the DNA samples. Mini preparations of the DNA were made which were simply a smaller scale variation of the above described bulk preparation of cosmid DNA. Briefly, *E. coli* NM554 containing cosmids were grown in L-broth in the presence of ampicillin overnight. The bacteria were harvested by centrifugation and lysed with a solution of 50 mM glucose, 25mM Tris.Cl (pH 8) and 10mM EDTA and then spun again. The pellet containing the bacteria contents was resuspended in a solution of 1% SDS and 0.2M NaOH by vortexing. The bacterial proteins and chromosomal DNA were precipitated with a solution of 5M potassium acetate and glacial acetic acid. The supernatant containing the cosmid DNA was mixed with a mixture of phenol, chloroform and isoamyl alcohol and then centrifuged. The DNA of the top liquid phase was precipitated with ethanol and collected by centrifugation. The pellet was air dried and resuspended in a solution containing Tris.Cl (pH8), EDTA and RNAase. A portion of this was mixed with buffer J (New England Biolabs) and restriction endonuclease Kpn 1 (New England Biolabs), that cuts DNA at the sequence displayed below (figure 2.10), and results in two overhangs called sticky ends, and the mixture was incubated for 2-12 hours at 37°C.
Figure 2.10. The recognition site and product of Kpn I endonuclease activity. The sequence recognised by Kpn I is shown. The comma ' denotes the site where the endonuclease cleaves leaving the sticky overhangs that are indicated by the underlined region.

The DNA fragments and a 1 Kb ladder (Gibco) were run on an 0.8% agarose gel containing ethidium bromide at 120 volts. The DNA bands were visualised under u.v. light. An Eppendorf transjector 5246 was used to microinject cosmid DNA into DRG neurones using sterile femtotips (Eppendorf).
Chapter 3

Ionic currents of Herpes simplex virus infected DRG neurones

Introduction

A Herpes simplex virus infection is known to affect the electrophysiological properties of excitable cells *in vitro* (Mayer & Westbrook, 1983; Fukuda & Kurata, 1981; Oakes *et al.* 1981; Avitabile *et al.* 1995). Adult Guinea pig DRG neurones infected with HSV-2 showed a marked loss in membrane excitability, as most infected neurones did not generate an action potential. When action potentials were fired they were characteristically broader and of a smaller amplitude than action potentials of
uninfected DRG neurones (Fukuda & Kurata, 1981). Action potentials evoked from infected DRG neurones in sodium free external solution showed a calcium spike comparable with those from uninfected DRG neurones. A loss of sodium conductance was suggested as responsible for the change of action potential shape after HSV infection (Fukuda et al. 1983). The first experiments described in this chapter repeat the previous experiments by measuring the membrane voltage in the current clamp mode in order to investigate the shape of action potentials of wt HSV 17\(^+\) infected adult rat DRG neurones.

The suggestion that the change in action potential shape was due to loss of sodium currents, however, could not be quantified by action potential analysis alone. Further experiments described in this chapter were designed to separate the ionic currents and to quantify any alterations observed. Firstly, the sodium currents of wt HSV 17\(^+\) infected DRG neurones were investigated. Secondly, the inwardly rectifying potassium current was investigated. An electrophysiological investigation of whole-cell potassium currents showed a virus strain dependent loss of the inwardly rectifying potassium current. Mayer (1986), observed that DRG neurones infected with a non-syncytial strain of HSV-1 showed a loss of the hyperpolarisation activated, inwardly rectifying potassium currents, whilst an infection with a syncytial strain did not alter these currents. The effect of an infection with a non-syncytial strain, such as wt HSV 17\(^+\), on the inwardly rectifying potassium currents of adult rat DRG neurones is described in this chapter. Finally, the outwardly rectifying potassium currents and calcium currents were investigated to give a fuller picture of the fate of ionic currents of wt HSV 17\(^+\) infected DRG neurones.

Before embarking on experiments to characterise any changes in ionic currents of wt HSV 17\(^+\) infected DRG neurones, it is first imperative to show that the neurones were actually virally infected. This was achieved in three ways, briefly described below. Firstly, indirect immunofluorescence, using an antibody directed against ICP4 (infected cell protein), was used to visualise infected neurones. The immediate early protein ICP4 is present in infected cells 24 hours after infection and is found in packaged virions (Yao & Courtney, 1989). Secondly, the HSV genome contains many
genes which are non-essential for viral replication; these genes may be removed and reporter genes can be inserted in their place. SAUS5-lacZ virus had a lacZ expression cassette inserted to interrupt the US5 gene of parental virus HSV-1 SC16 (Balan et al. 1994). Infection with SAUS5-lacZ and HSV-1 SC16 gave identical phenotypes, so the US5 gene (which encodes for glycoprotein J) was regarded as non-essential. Since infection with SAUS5-lacZ does not affect the rate of adsorption or penetration of virions, it is ideal for determining numbers of infected cells. When neurones were infected with SAUS5-lacZ the enzyme LacZ would be present and this reporter gene enzyme could turn the substrate X-gal deep blue such that the number of infected neurones in the population could be easily counted. Thirdly, the morphology of wt HSV 17+ infected DRG neurones was very distinct so that the percentage of infected neurones was again easily calculated.

In summary, the aim of this chapter was firstly to show that the entire population of DRG neurones were successfully infected with wt HSV 17+ and secondly to quantify any changes in sodium, potassium or calcium currents of the wt HSV 17+ infected adult rat DRG neurones.

Results

wt HSV 17+ infection of DRG neurones in culture

In order to investigate the electrophysiological properties of wt HSV 17+ infected DRG neurones it was first necessary to ensure that all the DRG neurones in culture were infected. In this study three methods were used to determine whether neurones were infected in vitro. In the first method indirect immunofluorescence with a monoclonal ICP4 antibody was used. Second, DRG neurones were infected with a SAUS5-lacZ, a mutant of HSV-1 containing a LacZ gene. Finally, the morphological changes of neurones after infection were examined.
Immunohistochemistry

Indirect immunofluorescence using a murine monoclonal ICP4 antibody was used to visualise DRG neurones infected with wt HSV 17+ at 4 and 24 hours post infection. Neurone cultures contain about 5% non-neuronal cells. Viral replication and lysis in non-neuronal cells is a potential source of virus. In order to show that the DRG neurones were infected directly upon addition of the virus and not indirectly from non-neuronal cell lysis, cells were fixed and visualised by indirect immunofluorescence before non-neuronal cell lysis could occur, at 4 hours post infection.

Populations of DRG neurones in culture were infected with wt HSV 17+ at 0.01, 0.1, 1, 5 and 50 p.f.u/neurone for 4 and 24 hours (p.f.u is the number of plaques formed per unit volume or weight of a virus suspension). The cells were then fixed and incubated with a murine monoclonal ICP4 antibody, washed and then incubated with an anti-mouse biotinylated antibody. The number of infected neurones were visualised subsequent to treatment with streptavidin conjugated fluorescein. The results are shown in figure 3.1. Table 3.1 shows that infecting DRG neurones with 5 or more p.f.u/neurone was sufficient to infect almost all the neurones in the culture. Photographs of infected DRG neurones are shown in figure 3.2.
Figure 3.1. Relationship between percentage of DRG neurone population infected and multiplicity of infection. Cells were infected with 0.01, 0.1, 1, 5 and 50 p.f.u/cell of wt HSV 17+. After (●) 4 hours and (■) 24 hours of infection the cells were fixed and immunolabelled; firstly with a murine monoclonal antibody which recognises ICP4 and secondly with an anti-murine biotinylated antibody, followed by streptavidin conjugated fluorescein to visualise the antibody. The percentage of the DRG neurone population with fluorescence was plotted against multiplicity of infection of wt HSV 17+.

<table>
<thead>
<tr>
<th>p.f.u/neurone</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of fluorescent neurones 4 hours/ total number of neurones</td>
<td>5/70</td>
<td>12/90</td>
<td>56/102</td>
<td>130/136</td>
<td>63/65</td>
</tr>
<tr>
<td>percentage of fluorescent neurones after 4 hours infection</td>
<td>7%</td>
<td>13%</td>
<td>55%</td>
<td>96%</td>
<td>97%</td>
</tr>
<tr>
<td>number of fluorescent neurones 24 hours / total number of neurones</td>
<td>5/150</td>
<td>7/60</td>
<td>40/84</td>
<td>87/90</td>
<td>50/50</td>
</tr>
<tr>
<td>percentage of fluorescent neurones after 24 hours infection</td>
<td>3%</td>
<td>12%</td>
<td>48%</td>
<td>97%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 3.1 The number and percentage of fluorescent neurones in a population of DRG neurones after infection for 4 or 24 hours, with various amounts of wt HSV 17+.
At 4 hours post infection with 5 or more p.f.u/neurone the majority of the neurone population was infected. This shows that DRG neurones were infected upon addition of virus to the media and not from viral replication in non-neuronal cells. It was therefore reasonable to assume that 5p.f.u/neurone of wt HSV 17+ was sufficient to infect all the neurones in the culture and that these were infected upon initial addition of the virus. Thus all the neurones in culture were at the same stage of viral replication.
**Infection with ΔUS5-lacZ**

DRG neurones were infected with ΔUS5-lacZ, a mutant HSV-1 virus which contains the reporter gene LacZ. LacZ encodes for an enzyme called β-galactosidase which can breakdown lactose into glucose and galactose. Infected neurones were detected by screening for the presence of β-galactosidase. A lactose analogue, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), can be broken down by β-galactosidase into a deep blue product which was easily visible in infected neurones. Figure 3.3 shows the result obtained when DRG neurones were infected with 0.01, 0.1, 1, 5 and 50 p.f.u/neurone with ΔUS5-lacZ after 24 hours. Photographs of DRG neurones infected with 1 p.f.u/neurone and 5p.f.u/neurone of ΔUS5-lacZ are shown in figure 3.4. Table 3.2 shows that addition of 5-50 p.f.u/neurone was sufficient to infect essentially the whole population of DRG neurones in the culture.

![Figure 3.3. Relationship between percentage of cell population infected and multiplicity of infection.](image)

**Figure 3.3.** Relationship between percentage of cell population infected and multiplicity of infection. Cells were infected with 0.01, 0.1, 1, 5 and 50 p.f.u/cell of ΔUS5-lacZ virus. At 24 hours after infection the cells were fixed and stained for the presence of β-galactosidase. The percentage of blue stained DRG neurones was calculated and plotted against multiplicity of infection.
<table>
<thead>
<tr>
<th>p.f.u/neurone</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>5</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of blue DRG neurones / total</td>
<td>8/225</td>
<td>15/195</td>
<td>81/305</td>
<td>149/161</td>
<td>145/152</td>
</tr>
<tr>
<td>percentage of blue neurones</td>
<td>4%</td>
<td>8%</td>
<td>27%</td>
<td>93%</td>
<td>95%</td>
</tr>
</tbody>
</table>

Table 3.2. The number and percentage of β-galactosidase expressing DRG neurones in a culture infected with various multiplicities of infection of SAUS5-lacZ.

Figure 3.4. Presence of β-galactosidase in SAUS5-lacZ infected DRG neurones. DRG neurone cultures were infected with a 1 p.f.u/neurone or b 5p.f.u/neurone of SAUS5-lacZ. After 24 hours of infection the cultures were treated with X-gal and the deep blue colour was visualised under a light microscope at x200.

Again these results demonstrate that 5p.f.u/neurone wt HSV 17* was sufficient to infect the majority of the neurones in the culture.
DRG neurone morphology

Figure 3.5a shows a control DRG neurone which has been in culture for 24 hours, with typical and extensive neurite growth. In contrast a DRG neurone from a culture infected with 5p.f.u/neurone wt HSV 17+ for 24 hours is shown in figure 3.5b. This was characteristic of wt HSV 17+ infected DRG neurones, which show rounded cell bodies and a lack of neurite outgrowth. This again showed that an infection with 5p.f.u/neurone wt HSV 17+ was sufficient to infect almost all of the population of DRG neurones in culture.

Figure 3.5. Morphological changes of DRG neurones after infection for 24 hours with wt HSV 17+. a An uninfected DRG neurone after 24 hours in culture with clearly visible neurite outgrowth. b A DRG neurone infected with wt HSV 17+ for 24 hours. The cell shows no neurite outgrowth and has a rounded appearance typical of infected DRG neurones.

Based on these studies 5p.f.u/neurone of wt HSV 17+ was used for all subsequent experiments to infect cultures of DRG neurones. Morphological examination for the presence or absence of extended neurites was used throughout these and subsequent electrophysiological studies as a rapid check of viral infection. If incomplete viral
infection was suspected the viability of the viral stocks was re-assessed by staining for ICP4 activity. New viral stocks were grown and titrated when required.

The excitability of wt HSV 17<sup>+</sup> infected DRG neurones

In this study the action potentials of DRG neurones infected with wt HSV 17<sup>+</sup> for 18, 24, and 40-48 hours were investigated. A control action potential from an uninfected neurone evoked with a depolarising current step of 300pA is shown in figure 3.6. A representative action potential from a wt HSV 17<sup>+</sup> infected DRG neurone evoked with a current injection of 400pA is shown in figure 3.7.

![Figure 3.6](image)

**Figure 3.6.** A representative action potential of an adult rat DRG neurone from a control, uninfected culture. The action potential was generated by a current injection of 300pA, from a resting membrane potential of -65mV, in the current-clamp mode.
Figure 3.7. A representative action potential from an adult rat DRG neurone from a culture infected wt HSV 17+ for 24 hours. The action potential was generated by a current injection of 400pA, from a resting membrane potential of -72mV, in current-clamp mode.

The longer duration (broadening) and reduced amplitude of the action potential in figure 3.7 is very distinct and typical of wt HSV 17+ infected DRG neurones. Even though the action potential from the wt HSV 17+ infected DRG neurone was generated from a more hyperpolarised resting potential than the control, so more sodium channels should have been available for opening, the overshoot was still lower than in control neurones.

Several characteristic features of the action potentials were measured for comparison, as shown in figure 3.8. The maximum rate of rise and fall of the action potential was calculated by differentiation, using the Pclamp6, clampfit software. Table 3.3 shows the mean results for action potentials from either control or wt HSV 17+ infected neurones at 18 hours, 24 hours or 40-48 hours post infection.
Figure 3.8. Measurements taken from action potentials. The properties of action potentials evoked in control and wt HSV 17+ infected DRG neurones were calculated for comparison. The width at -10 mV, maximum rate of rise, maximum rate of fall and overshoot were calculated using the clampfit software provided in the pclamp6, Clampfit suite of programs. The point of inflection was estimated by eye.
<table>
<thead>
<tr>
<th>time post infection</th>
<th>uninfected</th>
<th>18 hours</th>
<th>24 hours</th>
<th>40-48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>resting membrane potential (mV)</td>
<td>-57 ± 2</td>
<td>-54 ± 7</td>
<td>-47 ± 4</td>
<td>-52 ± 6</td>
</tr>
<tr>
<td>overshoot (mV)</td>
<td>38 ± 3</td>
<td>39 ± 5</td>
<td>26 ± 3**</td>
<td>14 ± 4**</td>
</tr>
<tr>
<td>width at -10mV (ms)</td>
<td>3.97 ± 0.26</td>
<td>6.88 ± 1.40</td>
<td>7.44 ± 0.83**</td>
<td>10.68 ± 2.10***</td>
</tr>
<tr>
<td>point of inflection (mV)</td>
<td>-21 ± 1</td>
<td>-14 ± 2</td>
<td>-14 ± 2</td>
<td>-14 ± 5</td>
</tr>
<tr>
<td>% neurones with an action potential</td>
<td>92%</td>
<td>89%</td>
<td>52%</td>
<td>47%</td>
</tr>
<tr>
<td>% neurones repetitively firing</td>
<td>33%</td>
<td>11%</td>
<td>31%</td>
<td>30%</td>
</tr>
<tr>
<td>max rate of rise (V/s)</td>
<td>73 ± 6</td>
<td>34 ± 6**</td>
<td>37 ± 7**</td>
<td>33 ± 9***</td>
</tr>
<tr>
<td>max rate of fall (V/s)</td>
<td>-33 ± 3</td>
<td>-16 ± 8</td>
<td>-11 ± 3***</td>
<td>-13 ± 4***</td>
</tr>
<tr>
<td>input resistance (mΩ)</td>
<td>121 ± 20</td>
<td>86 ± 26</td>
<td>84 ± 10</td>
<td>85 ± 50</td>
</tr>
<tr>
<td>capacitance (pF)</td>
<td>32.7 ± 2.7</td>
<td>30.9 ± 2.7</td>
<td>30.0 ± 2.2</td>
<td>24.7 ± 1.6</td>
</tr>
<tr>
<td>series resistance (mΩ)</td>
<td>5.3 ± 0.4</td>
<td>5.2 ± 0.6</td>
<td>5.2 ± 0.3</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>cell diameter (µM)</td>
<td>23 ± 1</td>
<td>25 ± 1</td>
<td>19 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>number of neurones</td>
<td>37</td>
<td>8</td>
<td>35</td>
<td>15</td>
</tr>
</tbody>
</table>

**Table 3.3. Comparison of action potential parameters from wt HSV 17+ infected and uninfected DRG neurones.** Measurements were made from DRG neurones held at a membrane potential of -60 mV. Mean ± s.e.m of action potential measurements and cell properties of DRG neurones 0 (uninfected), 18, 24 and 40-48 hours post infection with wt HSV 17+. *P<0.05, **P<0.02, ***P<0.001 for the wt HSV 17+ infected DRG neurones compared to the control, uninfected DRG neurones using Tukey's post hoc test following analysis of variance. For those parameters that were not significantly different, P>0.5. The values for these variables were normally distributed, as estimated by eye.
The results of this experiment show that wt HSV 17\(^+\) infected DRG neurones lose excitability and at 24 hours post infection only 52% of DRG neurones could fire an action potential. The action potentials of those infected neurones which were excitable were of a significantly different shape. Briefly, the width at -10 mV increased by more than double, the overshoot, the maximum rate of rise and fall of the action potential decreased, whilst the input resistance and resting membrane potential of these neurones remained unchanged.

The resting membrane potentials were corrected for junction potential errors by the addition of -7.3mV to each value recorded (see methods). The resting membrane potential of infected DRG neurones did not change significantly during wt HSV 17\(^+\) infection, which was a good indication that infected neurones were still alive and viable. The input resistance of the cell was measured by calculating the passive membrane voltage change with a small hyperpolarising or depolarising current injection. This value gives an indication of cell “leakiness” which was another good indicator of neurone viability. The neurones used in this comparison did not differ significantly in input resistance during wt HSV 17\(^+\) infection. The diameter and capacitance of the infected DRG neurones was not significantly different from the control, uninfected DRG neurones. This indicates that the DRG neurones tested came from populations of a similar neurone size distribution.

Although it is possible to speculate on the possible cause of the changes in action potential shape of wt HSV 17\(^+\) infected DRG neurones, it was not possible to make quantitative conclusions. For this reason, a more detailed voltage-clamp analysis was undertaken to investigate the contribution of each macroscopic current to the action potential changes that occurred during an infection with wt HSV 17\(^+\).
Sodium currents of DRG neurones infected with wt HSV 17^+

The changes in the shape of the action potential after 24 hours wt HSV 17^+ infection, particularly the significant decrease in the rate of rise and decreased overshoot, suggest that sodium conductance may be altered during the infection. In order to study this in a more quantitative manner, electrophysiological recordings in the voltage-clamp mode were made using internal and external solutions designed to isolate sodium currents (see methods).

Effect of wt HSV 17^+ infection on sodium currents of DRG neurones

The effect of a wt HSV 17^+ infection on the sodium currents of adult rat DRG neurones at various times during the lytic infection was investigated. The sodium currents were generated by stepping from a holding potential of -80mV to +10mV for 13msec. The currents evoked were normalised for neurone size (nA/pF). After 24 hours of infection many neurones had no sodium current at all (figure 3.9a), whilst others had small sodium currents. Figure 3.9b shows the mean normalised sodium current amplitudes at various time points during a wt HSV 17^+ infection of DRG neurones. The sodium current was significantly reduced in the DRG neurone population after 24 hours infection, which corresponds to the time that the action potential changed shape.
Figure 3.9. Temporal effects of wt HSV 17+ virus infection on normalised sodium currents in DRG neurones. a Raw sodium current traces evoked by voltage jump from -80mV to +10 mV for uninfected and wt HSV 17+ infected DRG neurones, 6-8 hours and 24-30 hours post infection. The leak current was subtracted from these traces. b The mean normalised sodium current ± s.e.m. for each population plotted against time post infection. Adult rat DRG neurones were infected with 5 p.f.u wt HSV 17+/ DRG neurone in vitro. At intervals during HSV infection sodium currents were recorded and normalised for neurone size. DRG neurones which were infected with wt HSV 17+ for 24-30 or 48 hours had significantly smaller normalised currents than neurones infected for less time if the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison was used, P<0.0001. The uninfected, 2, 6-8 and 16-20 hours post infection did not have significantly different normalised current amplitudes, P>0.8. The normalised current amplitudes of 24-30 and 48 hours post infection were not significantly different, P>0.9.
The reduction in the average normalised sodium current in wt HSV 17⁺ infected DRG neurones after 24 hours was partly due to the fact that many neurones had no sodium current at all. Figure 3.10 shows that the percentage of neurones with sodium currents decreased with duration of infection. At 24 hours post infection the normalised sodium current is dramatically reduced and only 28% cells had a measurable sodium current.

![Figure 3.10](image)

**Figure 3.10. The progressive loss of sodium currents during HSV infection in DRG neurones in vitro.** The sodium currents of DRG neurones were recorded at intervals after wt HSV 17⁺ infection. The percentage of cells with a sodium current were calculated and plotted against time post infection.

The finding that most wt HSV 17⁺ infected DRG neurones did not have a sodium current explains the loss of excitability of the infected DRG neurones. The DRG neurones that had a sodium current remaining after 24 hours of wt HSV 17⁺ infection were examined further to determine if these sodium currents were altered. The currents of wt HSV 17⁺ infected neurones were separated electrophysiologically into the TTX-S and TTX-R components. This was achieved by evoking currents with a depolarising step to -20 mV from two holding potentials of either -50 mV or -120 mV (see methods). After a prepulse to -120 mV, which removes the inactivation from
both TTX-S and TTX-R sodium channels, the total sodium current was evoked. After a prepulse to -50mV, which removes the inactivation from the TTX-R sodium channels only, a TTX-R current was evoked. The TTX-S current was obtained by subtracting the TTX-R current from the total sodium current using PClamp, clampfit software. The voltage protocol and the resultant traces are shown in figure 3.11a. The results of this analysis are shown in figure 3.11b for 13 wt HSV 17^ infected DRG neurones with a sodium current remaining and 28 uninfected DRG neurones. The results demonstrate that even when sodium currents remained after wt HSV 17^ infection for 24 hours, both the TTX-S and TTX-R components were significantly reduced in amplitude.
Figure 3.11. The TTX-S and TTX-R sodium currents of control and wt HSV 17+ infected DRG neurones. a Raw total sodium ($I_{Na}$), TTX-S and TTX-R sodium currents evoked by the voltage protocol illustrated from an uninfected DRG neurone. The leak currents were subtracted from these traces. b The normalised mean sodium current ± s.e.m from neurones with a measurable current (nA/pF). The remaining TTX-S and TTX-R sodium currents of wt HSV 17+ infected DRG neurones were significantly smaller than the TTX-S and TTX-R sodium currents of control cells (**P<0.05 Student t test).

The loss of sodium current generated by a step depolarisation from -80mV to +10mV could be explained by a shift in the sodium activation curve. To investigate whether there was a shift in the sodium current-voltage relationship of wt HSV 17+ infected DRG neurones, a family of sodium currents was evoked with 5 mV step depolarisations ranging from -50 mV to +25 mV after a prepulse to -120 mV that removed channel inactivation. Examples of families of sodium currents generated by this voltage protocol are displayed in figure 3.12a. wt HSV 17+ infected neurones with a sodium current remaining showed no visible shift in the current-voltage relationship as illustrated in figure 3.12b. The protocol used to investigate the sodium current-voltage relationship from infected neurones with a current remaining was also used to
examine neurones that showed no sodium current after the one step depolarisation voltage protocol. No sodium currents were revealed in these infected DRG neurones at any of the test potentials (-50 mV to +25 mV) after a prepulse to -120 mV.

Figure 3.12. The effect of wt HSV 17+ infection of DRG neurones on the sodium current-voltage relationship. a A family of sodium currents (with leak currents subtracted) from uninfected and wt HSV 17+ infected DRG neurones evoked by a range of test potentials between -50 mV and +25 mV after a prepulse to -120 mV. b The averaged mean normalised sodium current ± s.e.m plotted against command potential for control (●) n=16, and wt HSV 17+ infected DRG neurones (■) n=13.
Individual activation curves (fractional conductance plotted against test potential) were also constructed for the sodium currents remaining in infected neurones. The averaged data are plotted in figure 3.13 for wt HSV 17+ infected and control DRG neurones. The $V_{50}$ and slope factor values were also calculated for each neurone. The mean values calculated for each population of neurones tested are shown in table 3.4. There was no significant difference in the $V_{50}$ and slope factor values.

Figure 3.13. The sodium activation curve of control and wt HSV 17+ infected DRG neurones. The relationship between the normalised conductance and the command potential was determined from individual current-voltage relationships as described in materials and methods. The graph shows the mean normalised conductance ± s.e.m plotted against command potential for control (●) $n=16$, and wt HSV 17+ infected DRG neurones (■) $n=13$. The continuous lines were obtained by fitting a Boltzmann function to the normalised data.
Table 3.4. The effects of wt HSV 17+ infection of DRG neurones on the voltage dependence of the sodium conductance. $V_{50}$ refers to the potential ± s.e.m at which sodium conductance ($g$) is half of the maximum value ($g_{max}$). The slope factor of the normalised conductance-voltage relationship is a function of the voltage dependence of sodium conductances. $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between the data points of the conductance-voltage plots of each neurone. There was no significant difference between the $V_{50}$ or the slope factors of control or wt HSV 17+ infected DRG neurones with a sodium current remaining (Student t test, $P>0.5$).

<table>
<thead>
<tr>
<th></th>
<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of neurones</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-23.6 ± 1.4</td>
<td>7.4 ± 0.3</td>
<td>16</td>
</tr>
<tr>
<td>wt HSV 17+</td>
<td>-20.1 ± 1.3</td>
<td>9.1 ± 0.3</td>
<td>13</td>
</tr>
</tbody>
</table>

The steady-state inactivation properties of sodium currents from wt HSV 17+ infected and uninfected DRG neurones were analysed. Sodium currents were generated after a 420 msec prepulse to potentials in the range -120 to -5 mV which was immediately followed by a test pulse to 0mV for 10msec. The steady-state inactivation parameter ($h_{inact}$) was calculated by dividing the current evoked after a given prepulse by the maximum current evoked. Typical current traces from infected and uninfected DRG neurones are shown in figure 3.14a. The steady-state inactivation curve for wt HSV 17+ infected and uninfected DRG neurones were constructed by plotting $h_{inact}$ against the prepulse potential (figure 3.14b). The $V_{50}$ and slope factor values were measured for each neurone tested and the averaged data is shown in table 3.5. There was no significant difference between the electrophysiogical characteristics of uninfected and wt HSV 17+ infected DRG neurones.
Figure 3.14. The relationship between $h_a$ and the prepulse potential for wt HSV 17$^+$ infected or uninfected DRG neurones. a A family of raw current traces evoked by a test potential to 0mV after a prepulse ranging from -120 to -5mV from uninfected and wt HSV 17$^+$ infected DRG neurones. b The steady-state inactivation curve for uninfected DRG neurones (●) and for DRG neurones with a sodium current remaining after 24 hours infection with wt HSV 17$^+$ (■). The lines were obtained by fitting the mean normalised data with a Boltzmann function.
These rat neurones vary in size from 10-50 μM in diameter. The capacitance of all biological membranes is approximately 1μF/cm² and thus the area or size of neurones correlates to their capacitance. It was assumed that sodium channel density is constant among all DRG neurone subtypes and therefore normalising currents for neurone size were achieved by dividing by neurone capacitance (i.e. nA/pF). The possibility that there was a correlation between the loss of sodium current of wt HSV 17⁺ infected DRG neurones and neurone size was investigated by plotting sodium current amplitude against neurone capacitance (see figure 3.15a for wt HSV 17⁺ infected DRG neurones and figure 3.15b for uninfected DRG neurones).

<table>
<thead>
<tr>
<th></th>
<th>V₅₀ (mV)</th>
<th>slope factor (mV)</th>
<th>number of neurones</th>
</tr>
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<tbody>
<tr>
<td>control</td>
<td>-59.3 ± 6.7</td>
<td>8.3 ± 0.73</td>
<td>9</td>
</tr>
<tr>
<td>wt HSV 17⁺</td>
<td>-61.0 ± 6.6</td>
<td>8.1 ± 0.8</td>
<td>6</td>
</tr>
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</table>

Table 3.5. The average V₅₀ and slope factor values calculated from the steady-state activation curves plotted for each neurone tested. There was no significant difference between the V₅₀ or slope factor values of uninfected, control DRG neurones or wt HSV 17⁺ infected DRG neurones (Student’s t test, P>0.8.)
Figure 3.15. **The relationship between sodium current amplitude of a uninfected and b DRG neurones and neurone capacitance.**

a The sodium current amplitudes evoked from uninfected, control DRG neurones were plotted against neurone capacitance for a population of 105 neurones tested. There was a statistically significant correlation between the sodium current size and neurone capacitance, $P<0.005$. Correlations were examined by Pearson's statistical test, $r$ value = -0.7.

b The sodium current amplitudes evoked from wt HSV 17\(^+\) infected DRG neurones was plotted against neurone capacitance for a population of 106 neurones tested. There was no statistically significant correlation between the sodium current size and neurone capacitance, $P>0.1$. Correlations were examined by Pearson's statistical test, $r$ value = -0.02.

Figure 3.15 shows a correlation between size of sodium current and neurone capacitance. This correlation was a reflection of the fact that a larger neurone has a larger membrane with more sodium channels and thus will have a larger current. This correlation between sodium current amplitude and capacitance was not apparent for wt HSV 17\(^+\) infected DRG neurones, as all neurones either had no sodium current at all or a very small current. There was no correlation between neurone capacitance and loss of sodium currents of wt HSV 17\(^+\) infected DRG neurones. It is therefore not possible
to conclude that a particular size population of DRG neurones lose sodium conductance during a wt HSV 17^+ infection.

In summary, many wt HSV 17^+ infected DRG neurones did not have a sodium current. If they did have a sodium current then both the TTX-S and TTX-R components were dramatically reduced. This loss of sodium conductance could not be explained by a shift in the activation curve or steady-state inactivation curve of sodium currents during wt HSV 17^+ infection. The sodium conductance loss was not restricted to a particular size of DRG neurone.

Calcium and potassium currents of wt HSV 17^+ infected DRG neurones

The aim of these experiments was to investigate whether calcium and potassium currents were lost during a wt HSV 17^+ infection.

A sample of wt HSV 17^+ infected DRG neurones from each culture was investigated for the presence of sodium currents. The remaining neurones were used to record calcium channel currents or potassium currents. After showing that the neurones were infected, either by observing the characteristic morphology or by recording small or non existent sodium currents (only 33% cells had a sodium current), the other ionic currents were investigated.

Calcium currents

In these experiments barium was used as a charge carrier instead of calcium for several reasons. Calcium ions are permeable through sodium channels as well as calcium channels, whereas barium is much less permeable through sodium channels. This means that barium currents generated will pass only through calcium channels. Since
the aim of these experiments was to measure the presence and amplitude of calcium channel currents, rather than to analyse its inactivation properties, it was not a concern that the barium ions can inhibit the calcium-dependent inactivation of calcium channels. In fact, this was an additional reason for using barium as a charge carrier as barium currents can be recorded for longer and without the problem of calcium-dependent inactivation or rundown.

There are many types of calcium current present in DRG neurones. Calcium currents can be classified according to their voltage sensitivity, in particular, high-voltage activated (HVA) and low-voltage activated (LVA). The high-voltage activated currents are activated at high thresholds (>20mV) and inactivate at a slow rate (seconds) during a sustained depolarisation. Within this subset of calcium currents there are components which share similar activation kinetics, such as the L-type and N-type currents. The low-voltage activated current is activated at more negative potentials (~50mV) and inactivates rapidly. The main component of the LVA current is the T-type calcium current. The HVA currents can be recorded in isolation from LVA currents in two ways. First by holding the membrane potential level at a depolarised holding potential so that the LVA currents are not activated. Second, by inactivating most of the LVA channels with a depolarising pulse, followed by a larger depolarising pulse that evokes HVA current whilst the LVA channels are still inactivated. In this study the calcium currents were separated on the basis of their inactivation properties: the voltage protocol and typical current traces are illustrated in figure 3.16.

HVA type currents were evoked by a command potential from -100mV to -10mV for 50msec and LVA type currents by command potentials from -100mV to -50mV for 100msec. Figure 3.16b shows that the mean normalised HVA and LVA type calcium channel currents, with barium as the charge carrier of uninfected and wt HSV17+ infected DRG neurones, were not significantly different in size.
Figure 3.16. The effect of wt HSV 17+ infection of DRG neurones on HVA and LVA type calcium channel currents.  
a  Leak subtracted raw traces illustrating the separation of LVA and HVA calcium channel currents.  
b  The mean normalised peak HVA and LVA calcium current ± s.e.m of control or wt HSV 17+ infected DRG neurones for 24 hours.  
The HVA and LVA type calcium currents were not significantly different between the control and the wt HSV 17+ infected DRG neurones (Student t test, P>0.3).
Not all DRG neurones have a LVA calcium channel current; in fact, only 17% of the wt HSV 17\(^\text{+}\) infected and 26% of the uninfected DRG neurone population had measurable LVA currents in this experiment.

A family of barium currents was generated with a range of command potentials from \(-80\text{mV}\) to \(+40\text{mV}\). The calcium channel current-voltage relationship did not show an obvious shift during a wt HSV 17\(^\text{+}\) infection, as shown in figure 3.17.
Figure 3.17. Calcium current-voltage relationship of wt HSV 17+ infected or uninfected DRG neurones. a Two families of calcium channel currents evoked by a range of command potentials between -80mV and +40mV from an uninfected and wt HSV 17+ infected DRG neurone. The current traces shown have had the leak current subtracted. b The graph shows normalised calcium currents ± s.e.m plotted against the command potential from control (●) n=35, and wt HSV 17+ infected (■) n=27 DRG neurones.
The activation curves of the barium currents are shown in figure 3.18. Table 3.6 shows the $V_{50}$, voltage at which the conductance is half maximal, and the slope factor of the activation curves for wt HSV 17$^+$ infected and control DRG neurones. There was no significant difference in the electrophysiological characteristics of the barium conductance (through calcium channels)-voltage relationship of wt HSV 17$^+$ infected and control DRG neurones.

![Conductance-voltage relationship for calcium channels](image)

**Figure 3.18. Conductance-voltage relationship for calcium channels of control or wt HSV 17$^+$ infected DRG neurones.** The graph shows the mean normalised conductance ± s.e.m from control (●) n= 36, and wt HSV 17$^+$ infected (■) n=27 DRG neurones.

<table>
<thead>
<tr>
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<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of neurones</th>
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<tbody>
<tr>
<td>control</td>
<td>-16.9 ± 1.1</td>
<td>4.9 ± 0.5</td>
<td>36</td>
</tr>
<tr>
<td>wt HSV 17$^+$</td>
<td>-14.7 ± 1.5</td>
<td>4.6 ± 0.4</td>
<td>27</td>
</tr>
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</table>

**Table 3.6. Electrophysiological characteristics of voltage-gated calcium channel currents of control and wt HSV 17$^+$ DRG neurones.** $V_{50}$ and slope factor values of control and wt HSV 17$^+$ infected DRG neurones were not significantly different (Student t test P>0.1).
The inactivation parameter $h_\infty$ was calculated from the currents evoked by the following protocol; 320msec prepulse potential ranging from -100mV to +15mV, immediately followed by a 320msec command potential to -10mV, which evoked a maximal current. This voltage protocol and typical traces of control and wt HSV 17 infected DRG neurones are shown in figure 3.19a. $h_\infty$ was calculated by normalising the current generated by the prepulse with the maximal current generated by the test potential. Figure 3.19b illustrates that there is no difference in the steady-state inactivation of calcium channel currents of control and wt HSV 17 infected DRG neurones.
Figure 3.19. The effect of prepulse potential on calcium currents from control or wt HSV 17+ infected DRG neurones. a The steady-state inactivation voltage protocol used to evoke the family of calcium channel currents for control, uninfected and wt HSV 17+ infected DRG neurones. b The graph shows normalised calcium currents ± s.e.m plotted against the prepulse potential from control (●) n=9, and wt HSV 17+ infected (■) n=8 DRG neurones. The continuous lines were obtained by fitting a Boltzmann function to the mean normalised data.

<table>
<thead>
<tr>
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<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of neurones</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-48.2 ± 0.7</td>
<td>14.6 ± 0.8</td>
<td>9</td>
</tr>
<tr>
<td>wt HSV 17+</td>
<td>-45.6 ± 0.9</td>
<td>13.2 ± 0.2</td>
<td>8</td>
</tr>
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Table 3.7. The steady-state inactivation electrophysiological characteristics of calcium channel currents of uninfected or wt HSV 17+ infected DRG neurones. There was no significant difference between the $V_{50}$ or slope factor values (Student t test, $P>0.6$).
In contrast to the sodium currents which were lost 24 hours after infection, the calcium channel currents of wt HSV 17^+ infected DRG neurones remained unchanged in amplitude, activation and steady-state inactivation.

**Potassium currents**

It is possible that the broadening of the action potential at -10mV was due to an absence of delayed rectifier type potassium currents such that the membrane slowly repolarised as sodium or calcium channels inactivated. It was interesting to investigate whether the potassium channels were affected by the wt HSV 17^+ infection. The potassium currents of DRG neurones can be classified into two types: the delayed rectifier type which activate upon depolarisation and the inward rectifier type which activate only when the membrane hyperpolarises.

**Delayed rectifier type potassium currents**

There is a large variability in the kinetic properties of voltage-gated outward currents in different DRG neurones. The delayed rectifier type potassium currents can be crudely separated into two groups, inactivating and non-inactivating, based on their inactivation properties. Total potassium currents were evoked from -80mV with a step potential to +10mV and the non inactivating potassium currents were evoked after a prepulse to -50mV from a test potential of +10mV. The inactivating potassium current could be separated by subtraction. The voltage protocol and a typical trace is shown in figure 3.20a. Figure 3.20b and c shows that there was no significant difference in the normalised amplitude of the non-inactivating and inactivating delayed rectifier type potassium current.
Figure 3.20. The effect of HSV infection of DRG neurones on inactivating and non-inactivating potassium currents. 

a Inactivating and non-inactivating delayed rectifier type potassium currents from an uninfected DRG neurone (leak subtracted). The total potassium currents were evoked from -80mV with a step potential to +10mV and the non-inactivating potassium currents were evoked after a prepulse to -50mV with a test potential of +10mV. The inactivating potassium current could be separated by subtraction. 

b The normalised mean non-inactivating potassium current amplitude ± s.e.m is shown for control and wt HSV 17\textsuperscript{r} infected DRG neurones. There was no significant difference between the normalised potassium current amplitude of control or infected DRG neurones (Student t test, P>0.3). 

c The normalised inactivating type potassium current amplitudes of infected and control DRG neurones. There was no significant difference between the normalised current amplitude of control or infected DRG neurones (Student t test, P>0.2).
A family of outward potassium currents in DRG neurones was evoked from a holding potential of -80mV by a series of 15ms depolarising voltage steps between -90mV and +40mV. The membrane was then stepped back to -40mV. The tail currents upon stepping back to -40mV are displayed in figure 3.21a. On stepping to -40mV the current falls to an instantaneous value which was proportional to the number of channels open immediately prior to the step. The tail currents were measured and normalised with respect to maximum tail current, and then plotted against the voltage prior to the -40mV step, giving the activation curve as shown in figure 3.21c.

To analyse the steady-state inactivation properties of the outward potassium currents, a family of currents was evoked at +45mV after a 1s prepulse to membrane potentials ranging from -120mV to 0mV (figure 3.21b). Activation of outward currents can be seen for the last few prepulse potentials. The activation and steady-state inactivation curves of the delayed rectifier type potassium currents are shown in figure 3.21c. There was no apparent change in the voltage dependence of activation or inactivation of wt HSV 17+ infected or control DRG neurones. There was no significant difference in the slope factors and V_{50}, voltage at half maximal conductance, as shown in table 3.8.
Figure 3.21. The activation and steady-state inactivation of control or wt HSV 17+ infected DRG neurones. 

a A family of outward potassium currents in DRG neurones. Neurones were held at -80mV. The activation protocol consisted of a series of 15ms depolarising voltage steps between -90mV and +40mV. Note the outward tail currents that occur when the membrane is stepped back to -40mV. 

b The steady-state inactivation of outward potassium currents were evoked at +45mV after a 1s prepulse at membrane potentials ranging from -120mV to 0mV. Activation of outward currents can be seen for the last few prepulse potentials. Leak currents were subtracted. 

c The activation and steady-state inactivation curves of control or wt HSV 17+ infected DRG neurones. The graph shows the normalised mean conductance ± s.e.m plotted against the command potential. The activation and steady-state inactivation curves of control (●) n=25, and wt HSV 17+ infected (■) n=11 DRG neurones, are shown. The curves are obtained by fitting the data points with a Boltzmann function.
### Table 3.8. Electrophysiological characteristics of the delayed rectifier type potassium conductances of control or wt HSV 17\(^+\) infected DRG neurones.

<table>
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<th></th>
<th>Activation</th>
<th>Steady-State inactivation</th>
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<tbody>
<tr>
<td></td>
<td>(V_{50}) (mV)</td>
<td>slope factor (mV)</td>
</tr>
<tr>
<td>control</td>
<td>-26.8 ± 1.9</td>
<td>12.8 ± 1.3</td>
</tr>
<tr>
<td>wt HSV 17(^+)</td>
<td>-23.7 ± 1.7</td>
<td>17.3 ± 2.7</td>
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\(V_{50}\) refers to the potential ± s.e.m at which conductance (g) is half of the maximum value (gmax). The slope factor is a function of the voltage dependence of the conductances. The \(V_{50}\) and slope factor values were calculated from Boltzmann curves fitted between the data points from each cell. There was no significant difference between the \(V_{50}\) and slope factor values of the activation curves (Student t test, \(P>0.07\)). There was also no significant difference between the \(V_{50}\) and slope factor values of the steady-state inactivation curves of infected and uninfected DRG neurones (Student t test, \(P>0.1\)).

### Inward rectifier type potassium currents

Inward rectifier potassium channels act like diodes which favour an inward flow of potassium ions during hyperpolarisation and very little ion flow during depolarisation. For cells that express predominantly inward rectifier channels, extended periods of depolarisation can be maintained, which is important for the generation of long action potentials. The small outward conductance of the inward rectifier channels at membrane potentials just above the potassium reversal potential (\(E_k\)), which is about -90mV, helps to maintain the resting membrane potentials close to \(E_k\). Thus the inward rectifier channels play a role in maintaining neurone resting membrane potential and in controlling excitability. A typical inwardly rectifying potassium current can be seen in figure 3.22b.

Figure 3.22c shows the mean normalised amplitude of the inwardly rectifying potassium current generated from a 900msec hyperpolarising step to -120mV, from a
holding potential of -70mV. There was no significant difference in the current amplitudes of the inwardly rectifying potassium current of uninfected or wt HSV 17\textsuperscript{+} infected DRG neurones. The presence of the inwardly rectifying potassium currents were also observed by clamping the neurones in the current clamp mode and eliciting a family of voltage traces with a series of hyperpolarising current steps. When inward rectifier currents were expressed in DRG neurones the characteristic time-dependent rectification was observed, as in figure 3.22a.

![Image A](image.png)

20mV
100ms

![Image B](image.png)

0.5nA
100ms
Figure 3.22. A comparison of the inwardly rectifying, potassium current amplitude, normalised for neurone size of control or wt HSV 17+ infected DRG neurones. a A family of voltage traces, elicited by 900ms hyperpolarising current steps from a wt HSV 17+ infected DRG neurone, showing time dependent rectification. b An inwardly rectifying potassium current evoked from a wt HSV 17+ infected DRG neurone, following a step from -70mV to -120ms, for 900ms. c The graph shows the mean normalised sustained current amplitude ± s.e.m of control or wt HSV 17+ infected DRG neurones. There was no significant difference between the normalised current amplitudes (Student t test, P>0.1).

A family of inwardly rectifying potassium currents were evoked from a holding potentials of -70mV, with step depolarisations from -50mV to -140mV. The tail current amplitudes were measured upon membrane repolarisation to -70mV, normalised with respect to peak tail current and then plotted against command potential. The activation curve is illustrated in figure 3.23, and shows the activation curves of wt HSV 17+ infected and uninfected DRG neurones almost overlap. This indicates no obvious change in the activation of inwardly rectifying potassium channels during a wt HSV 17+ infection. The electrophysiological characteristics, calculated from such curves fitted with a Boltzmann function for each neurone tested,
are shown in table 3.9. Statistical analysis showed that there was no significant difference in the slope factor and $V_{50}$ values.

\[ \text{wt HSV infected} \]
Figure 3.23. Activation curves for the hyperpolarisation activated conductances recorded from control and HSV infected DRG neurones. 

(a) A family of inwardly rectifying potassium currents evoked by a range of potentials between -50mV and -140mV. 

(b) The normalised mean inward tail current amplitude ± s.e.m recorded on repolarisation to the holding potential, plotted against the hyperpolarising command potential. The activation curve of control (●) n=9, and wt HSV 17+ infected (■) n=7 DRG neurones are shown. The activation curves shown are obtained by fitting the mean normalised data to a Boltzmann function.

<table>
<thead>
<tr>
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<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of cells</th>
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<tbody>
<tr>
<td>control</td>
<td>-94.1 ± 1.4</td>
<td>10.6 ± 1.8</td>
<td>9</td>
</tr>
<tr>
<td>wt HSV 17+</td>
<td>-91.2 ± 1.6</td>
<td>8.7 ± 0.7</td>
<td>7</td>
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Table 3.9. The slope factor and $V_{50}$ from the activation curves of hyperpolarisation activated conductance in control or wt HSV 17+ infected DRG neurones. There was no significant difference between the $V_{50}$ and slope factor values of the inwardly rectifying potassium channel activation curves (Students’s t test, P>0.08).
Inwardly rectifying potassium channels are not uniformly distributed throughout a population of DRG neurones, and are predominantly found in the larger DRG neurones. The distribution of the inwardly rectifying potassium currents of DRG neurones was not altered during a wt HSV 17\textsuperscript{+} infection, as shown in figures 3.24 and 3.25.

**Figure 3.24.** The differential distribution of inwardly rectifying potassium current expression with respect to capacitance in control neurones. The \( \backslash \ll / \) shading indicates those cells expressing inwardly rectifying potassium current, while those that did not express a current are indicated by \( \backslash \ll / \) shading. The total number of cells tested was 38, bin size 10 pF.
Figure 3.25. The differential distribution of inwardly rectifying potassium current expression with respect to HSV infected cell capacitance. The \<//> shading indicates those cells expressing inwardly rectifying potassium current, while those that did not express a current are indicated by |||| shading. The total number of cells tested was 26, bin size 10 pF.

In summary, the outward, delayed rectifier type potassium conductances were unaltered 24 hours after wt HSV 17\(^{+}\) infection of DRG neurones. The inwardly rectifying potassium current was also unaffected by the infection. This is in contrast to the sodium conductance which was dramatically reduced in DRG neurones which had also been infected with wt HSV 17\(^{+}\). wt HSV 17\(^{+}\) infection of DRG neurones specifically causes a loss in sodium conductance and not other ionic currents.
Discussion

A loss of excitability was noted in wt HSV 17\textsuperscript{+} infected DRG neurones after 24 hours of infection. When neurones did fire an action potential, its shape was broader and of a reduced amplitude, compared to those of the uninfected neurones. These findings were consistent with a previous report which showed that an infection of adult Guinea pig DRG neurones with HSV-2 resulted in a similar action potential shape change (Fukuda & Kurata, 1981).

Analysis of the action potential shape after wt HSV 17\textsuperscript{+} infection for 24 hours gives clues as to which ionic currents may have contributed to the change in action potential shape. For example, the rate of rise of the action potential, which is a function of sodium inward current (Huxley & Hodgkin, 1952), was decreased by about a half in the infected DRG neurones. This finding suggests that a sodium current loss may underlie the change in the shape of the action potential. Another clue which suggested the involvement of reduced sodium conductance in action potential shape change was the significant decrease in overshoot or action potential amplitude. The action potentials of infected DRG neurones reached a less depolarised peak membrane potential than those of control neurones, and this would have the knock on effect of failing to activate as many delayed rectifier type potassium channels. Thus resulting in a slower repolarising phase of the action potential. This was consistent with the observations that the repolarising phase of the action potential of HSV infected DRG neurones were significantly slower than those of the controls. Calcium currents inactivate during the falling phase of the action potential and the slower rate of fall of the action potential could result from calcium current inactivation, giving the action potential the characteristic “hump” shape seen with HSV infection. In short, it is not possible from action potential analysis alone to discover which electrophysiological changes underlie the action potential shape change or loss of excitability during a wt
HSV 17\(^+\) infection. This question can be answered by investigating individual whole-cell currents in the voltage-clamp mode.

The sodium current amplitudes of DRG neurones were measured at various different time points during an infection with wt HSV 17\(^+\). After 24 hours infection, many DRG neurones had no sodium current at all, and the remaining sodium currents were of a dramatically reduced amplitude. Further investigation showed that at 24 hours post infection both the TTX-S and TTX-R sodium currents were reduced in amplitude compared to the currents of uninfected DRG neurones. It is interesting to note that the remaining sodium current was not predominately TTX-R, which is usually smaller in amplitude than the TTX-S current amplitude. The remaining current was not found in any particular size of DRG neurones. It is also worth noting that neurones that did not show a sodium current with the one step depolarisation protocol from -80mV to +10mV, did not subsequently reveal a current when a prepulse to a potential of -120 mV was used to remove inactivation of the all sodium channels. The current-voltage relationship, did not reveal a shift in the activation curve which could have explained an apparent loss of sodium current at +10mV.

The HVA and LVA type calcium channel current amplitudes were not significantly different from infected and uninfected DRG neurones. This indicated that there was no loss of current comparable to the reduction in sodium current amplitudes in DRG neurones infected with wt HSV 17\(^+\). The activation and inactivation curves were also unaffected. The experiments were performed using barium as a charge carrier, which has some disadvantages, as listed below. Barium has a different permeability to calcium through different types of calcium channels. T-type channels pass barium as well as calcium, but L- and N-type channels pass barium about twice as well as calcium. Barium also slows the inactivation of L- and N-type channels, but not T-type channels (Bean, 1985). The use of barium to compare current amplitudes of HVA and LVA currents between experimental conditions was considered valid since the purpose of the experiment was to investigate the presence and comparative size of the currents from infected and uninfected neurones, rather than to characterise their detailed biophysical properties. Initial experiments were performed with calcium as the charge carriers.
carrier, simply to show the presence of calcium currents. The calcium channel currents were not pharmacologically separated into subtypes as the less time consuming electrophysiological separation of currents was sufficient to show that there was no gross loss of calcium currents comparable with the loss of sodium current in wt HSV 17^+ infected DRG neurones.

The delayed outward rectifier type potassium currents, both inactivating and non inactivating, showed no significant difference in amplitude between wt HSV 17^+ infected and uninfected DRG neurones. The activation and steady-state inactivation curves of the total delayed rectifier type potassium currents also showed no significant difference between wt HSV 17^+ infected and uninfected DRG neurones, which is consistent with the previous observations (Mayer, 1986). The results of the experiments investigating the inward rectifier type potassium currents, however, were not consistent with previous findings. Mayer (1986), showed that when DRG neurones were infected with non-syncytial strains of HSV-1, the inwardly rectifying type potassium currents were lost, whereas neurones infected with syncytial strains of HSV-1 showed hyperpolarisation activated currents comparable with those of uninfected neurones. The results described here show that DRG neurones infected with the non-syncytial strain HSV 17^+ (Brown et al. 1973) still had inwardly rectifying potassium currents of comparable amplitude to those of control, uninfected DRG neurones. It has recently been shown that the inwardly rectifying potassium currents are predominately found in large diameter cell bodies and that the current is of a higher density in the larger neurones compared with smaller neurones with an inwardly rectifying current (Scroggs et al. 1994). Mayer only tested 5 HSV 17^+ DRG neurones and no indication was given of neurone size. It is therefore possible that inwardly rectifying potassium currents may have been observed if a larger population had been tested.

In summary, the results in this chapter showed that infection of neuronal cultures with 5p.f.u/neurone wt HSV 17^+ was sufficient to infect a very high proportion of neurones in the culture. After 24 hours of infection there was a dramatic loss of sodium current which reflected a loss of sodium conductance rather than a shift in the activation or
inactivation kinetics of the sodium channels. Many (~70%) infected DRG neurones had no sodium current, and if there was a sodium current remaining then both the TTX-S and TTX-R sodium currents were reduced in amplitude. This loss of sodium conductance could certainly explain the loss of excitability of DRG neurones which had been previously reported (Mayer, 1986; Oakes et al. 1981; Fukuda & Kurata, 1981). In comparison, however, the calcium and potassium currents of the wt HSV 17观看 infected DRG neurones were unaffected after 24 hours infection. The aspects of the viral infection which caused the selective loss of sodium current from the infected DRG neurones and the possible underlying mechanism were studied next. The results of experiments designed to investigate these questions are discussed in subsequent chapters.
Chapter 4

Aspects of viral infection

Introduction

DRG neurones lose excitability after 24 hours of infection with wt HSV 17+. This is the result of a dramatic decrease in sodium conductance as described in chapter 3. In this chapter an attempt has been made to determine which aspects of wt HSV 17+ infection are responsible for the loss of sodium conductance in DRG neurones. In classical virology, the function of various components of the life cycle of HSV-1 have been studied using combinations of viral mutants together with biochemical agents which block specific biological processes. The question is, what facet of viral infection elicits the dramatic decrease in sodium current amplitude?
The wt HSV 17+ virion contains many proteins, such as the capsomer proteins, tegument proteins and glycoproteins. It is possible that simply virion entry into DRG neurones may be sufficient to cause the loss of sodium conductance. A virion protein, perhaps a glycoprotein, could act directly by blocking sodium channels, or indirectly by triggering a sodium channel down regulation mechanism.

The virion tegument protein, virus host shutoff protein (vhs), induces rapid degradation of cellular mRNAs as well as the shutoff of most host cell protein synthesis upon viral entry (Fenwick & McMenamin, 1984). Such general repression of DRG neurone protein synthesis may indirectly cause the loss of sodium conductance. A factor involved in sodium channel function or the sodium channels themselves may rapidly turnover. Depletion of host proteins does occur during wt HSV 17+ infections, a specific example being the reduction of the DNA-dependent protein kinase (DNA-PK) levels (Lees Miller et al. 1996). A perturbation in host cell protein synthesis may be sufficient to deplete levels of a protein with a short half life involved in sodium channel function, hence the loss of sodium current.

The loss of sodium conductance in infected DRG neurones could also be the result of viral proteins synthesised during the course of infection. Upon entry of viral DNA into the nucleus the first set of genes to be expressed in the temporal cascade of viral gene expression are the immediate early genes (Honess & Roizman, 1974). There are five immediate early genes encoding viral proteins, ICP0, ICP4, ICP27, ICP22 and ICP47 (Pereira et al. 1977). They are transcribed and translated by cellular machinery and do not require prior viral protein synthesis (Honess & Roizman, 1974; Costanzo et al. 1977). ICP22 and ICP47 do not affect viral gene expression (Everett, 1984). In contrast ICP0, ICP4 and ICP27 are involved in regulating immediate early, early and late gene expression (O-Hare & Hayward, 1985). A summary of the immediate early genes ICP0, ICP4 and ICP27 are listed below.
ICP0 is a 110kDa nuclear phosphoprotein, also known as Vmw110 and is the product of immediate early gene 1 (Pereira et al. 1977). Transient expression assays show ICP0 is a promiscuous transactivator of viral and non-viral promoters (Everett, 1984). In the absence of ICP0, virus replication is inefficient or sluggish, with a lower probability of initiating a productive infection. These defects can be overcome by increasing the multiplicity of infection (Stow & Stow, 1986). Mutant viruses with deletions in the gene encoding ICP0 are unable to reactivate from explanted sensory ganglia harboring latent virus, which suggests that ICP0 plays a role in the switch between lytic and latent state (Leib et al. 1989). The phenotype of cells infected with mutant HSV, with deletions in both genes encoding ICP0 and ICP4, can be rescued by transfection with plasmids encoding either ICP0 or ICP4, as both can activate immediate early, early and late gene expression. The levels of expression, however, are much greater when both plasmids encoding ICP0 and ICP4 are transfected together. This suggests that the activity of ICP0 is synergistic with ICP4 (Everett, 1984). Although ICP0 is non-essential for viral growth, it does play a role in efficient initiation of viral gene expression probably by stabilising viral proteins by binding to HAUSP (herpesvirus associated ubiquitin specific protease) (Everett et al. 1997).

ICP4 (expressed from immediate early gene 3) has an apparent molecular weight of 175 kDa as estimated by SDS-PAGE, is a phosphoprotein and is located in the nucleus of infected cells (Courtney & Benyesh Melnick, 1974; Pereira et al. 1977). ICP4 is packaged into the tegument of virions (Yao & Courtney, 1989). ICP4 binds DNA as a homodimer, and is found in solution as a multimer or dimer, (Michael & Roizman, 1989; Metzler & Wilcox, 1985). Of the five immediate early genes, ICP4 is perhaps the most important. Studies with temperature sensitive mutants which map to ICP4, show an absence of viral growth through failure to activate early and late promoters at non-permissive temperatures (Watson et al. 1979). In addition, experiments with temperature sensitive mutants (which map to ICP4) showed that ICP4 autoregulates its own synthesis and represses the expression of other immediate early genes (DeLuca & Schaffer, 1988; Michael & Roizman, 1993). For this reason an overproduction of immediate early proteins, but no expression of early or late genes, occurs during an infection with virus lacking functional ICP4 (DeLuca et al. 1984). As described
above, transient expression assays show that ICP0 and ICP4 have synergistic enhancing effects on early and late gene expression. In contrast, ICP27 can exert a positive or negative effect on ICP4 induced gene expression (Sekulovich et al. 1988).

ICP27 (gene product of immediate early gene 2) is a nucleophosphoprotein, with an apparent molecular weight of 63 kDa as estimated by SDS-PAGE (Wilcox et al. 1980; Knipe et al. 1987; Ackermann et al. 1984). Null ICP27 mutants do not replicate and show a decrease in viral DNA synthesis and late gene expression, whilst immediate early proteins accumulate (Rice & Knipe, 1990). ICP27 is involved in the switch between early and late gene expression and is absolutely essential for formation of infectious progeny (Sacks et al. 1985; McCarthy et al. 1989). Experiments have shown that ICP27 can either activate or repress expression of cotransfected reporter genes in uninfected cells (Rice et al. 1989; Everett, 1986). Activation of reporter genes has been correlated with the presence of particular polyadenylation signals, while repression has been correlated with the presence of introns (Cai & Schaffer, 1989). The range of regulatory feats performed by ICP27 includes stimulation of DNA synthesis, post transcriptional destabilisation of immediate early gene RNA and inhibition of RNA splicing (Smith et al. 1992; Leary et al. 1989). The inhibition of host cell RNA splicing is thought to play a role in secondary shutoff of host protein synthesis (Hardwicke & Sandri Goldin, 1994).

After the expression of immediate early and early genes the virus begins to replicate its DNA and express late genes. Early genes encode for proteins that are involved in DNA replication and the late proteins encode for virion structural proteins. Arresting this phase of the cycle with a specific HSV DNA replication inhibitor, acyclovir, prevents the expression of true late genes (Holland et al. 1980). Since early gene expression is inhibited by late genes, the presence of acyclovir results in enhanced early gene expression.
The same tools which have been designed to dissect the life cycle of HSV-1 can be used to determine the components responsible for loss of sodium conductance in the host DRG neurones. The experiments described in this chapter use the chemical agents cycloheximide to investigate the role of virion components, and acyclovir to investigate the role of viral DNA synthesis in the loss of sodium conductance. HSV-1 mutants lacking functional immediate early genes were used to study their effect on electrophysiological changes during an infection.

Finally, a cosmid library of wt HSV 17\(^+\) genome was used in an attempt to discover the gene(s) encoding the causative proteins involved in the loss of sodium conductance. The wt HSV 17\(^+\) genome is very large, consisting of 152 Kb and encoding at least 70 distinct proteins (McGeoch \textit{et al.} 1988). It is possible that a single protein could cause the loss of sodium conductance in wt HSV 17\(^+\) infected DRG neurones. Five fragments together comprising the wt HSV 17\(^+\) genome were available in a cosmid library (Cunningham & Davison, 1993). Microinjection of wt HSV 17\(^+\) genome fragments was used to locate possible gene products responsible for the loss of sodium conductance.

In summary, the aim of the studies reported in this chapter was to examine the possible features of viral infection that cause the loss of sodium conductance; virus entry, viral immediate early gene expression, and viral DNA replication. More specifically, the role of expression of specific viral genes was investigated using a cosmid library of wt HSV 17\(^+\) genome.
Results

Virus entry

The virion host shutoff protein

The virion particle contains many different proteins, including the virion host shut off protein (vhs). Vhs enters DRG neurones upon viral entry and inhibits host cell protein synthesis (Sydiskis & Roizman, 1966). This acts to subvert neuronal protein synthesis machinery to produce viral proteins. The protein synthesis inhibitor, cycloheximide, was used to mimic the host protein synthesis shutoff induced by vhs, to investigate whether inhibition of protein synthesis reproduced the effects of a wt HSV 17 infection.

Cycloheximide inhibits translocase, which catalyses the transfer of elongating proteins from the P site to the A site of ribosomes (Obrig et al. 1971). Cycloheximide has previously been used to inhibit protein synthesis in DRG neurones at 50μg/ml (Oakes et al. 1981). The degree of protein synthesis inhibition in DRG neurones in the presence of 50μg/ml of cycloheximide was first investigated.

DRG neurones were treated with 50μg/ml of cycloheximide for 24 hours in the presence of L-[35S]methionine. Radiolabelled amino acid, methionine, would be incorporated into any proteins synthesised. The levels of radiolabeled proteins were measured by detecting the radioactivity incorporated into precipitated cell proteins. Figure 4.1 shows that proteins precipitated from control cells have a mean of 338051 decays per minute, but protein precipitated from cells treated 50μg/ml of cycloheximide had a reduced mean of 11447 decays per minute. This indicates that incubation of DRG neurones for 24 hours in the presence of 50μg/ml of cycloheximide is sufficient to inhibit protein synthesis by 97%. The DRG neurone cultures treated with cycloheximide showed no obvious signs of cell death.
**Figure 4.1. Inhibition of protein synthesis in DRG neurones by incubation with 50μg/ml cycloheximide for 24 hours.** DRG neurones were incubated in medium containing L-[35S]methionine in the presence or absence of the protein synthesis inhibitor cycloheximide for 24 hours. Cell proteins were precipitated, collected on filters and radioactive emission was determined. The levels of L-[35S]methionine were counted using a liquid scintillation counter. The graph shows the mean decays per minute ±s.e.m from cell proteins with L-[35S]methionine incorporated (** P<0.0001, Student’s t test).

To investigate whether vhs proteins cause the loss of sodium currents simply by inhibiting protein synthesis, neurones were treated with cycloheximide for 24 hours followed by electrophysiological recording of the sodium currents.

Sodium currents were generated by a depolarising step from a holding potential of -80mV to +10mV for 13 msec from, wt HSV 17+ infected, uninfected neurones and DRG neurones treated with cycloheximide for 24 hours. The step depolarisation from -80mV to +10mV will activate some TTX-S as well as TTX-R sodium currents i.e. total sodium current. (This protocol will be referred to as the one step depolarisation protocol throughout the rest of this chapter.) All control and cycloheximide treated cells showed a measurable sodium current (typical traces, figure 4.2a). Figure 4.2b shows that the normalised current (nA/pF) of DRG neurones treated with 50 μg/ml cycloheximide for 24 hours was not reduced compared to the currents of control DRG
neurones. This indicates that merely inhibiting protein synthesis to mimic the effect of the vhs proteins does not explain the observations with wt HSV 17^+ infected DRG neurones. The sodium current amplitudes normalised for cell size (nA/pF) of cycloheximide treated DRG neurones were not significantly different from those of the uninfected neurones. The normalised sodium current amplitude of wt HSV 17^+ infected DRG neurones in this experiment was, however, significantly smaller than those from both cycloheximide and uninfected neurones in accordance with the results in chapter 3.

![Diagram](image)
Figure 4.2. Comparison of protein synthesis inhibition and wt HSV 17+ infection in DRG neurones. **a** Sodium current traces of uninfected, cycloheximide treated and wt HSV 17+ infected DRG neurones evoked from a holding potential of -80mV by a depolarising step to +10mV. The leak currents were subtracted. **b** The graph shows normalised peak sodium current ±s.e.m from, control cells, cells after infection with wt HSV 17+ or treatment with 50µg/ml cycloheximide for 24 hours. Normalised sodium currents from both control and cycloheximide treated cells were significantly different from the currents from wt HSV 17+ infected cells (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, **P<0.02). The normalised currents from control and cycloheximide treated cells were not significantly different from each other P>0.2.

Data illustrating the voltage dependence of sodium currents evoked from DRG neurones treated with cycloheximide for 24 hours is presented graphically and tabulated together with the results from another experiment investigating the effects of different virion proteins (see page 124). Sodium currents were generated after a hyperpolarising pulse to -120mV, to remove inactivation, by a series of step depolarisations with 5mV increments to test potentials between -50mV and +25mV. The peak currents generated were plotted against test potential. The current-voltage
relationships for each uninfected, cycloheximide treated and wt HSV 17\(^+\) infected cells were normalised to maximum current. The averaged data is displayed in figure 4.4. Individual activation curves (fractional conductance plotted against test potential) were also constructed. The averaged activation curves are plotted as shown in figure 4.5 for uninfected, cycloheximide treated and wt HSV 17\(^+\) infected cells and the calculated \(V_{50}\) and slope factors are displayed in table 4.1 (page 124). There was no significant difference in the \(V_{50}\) and slope factors of sodium currents generated from uninfected, cycloheximide treated and wt HSV 17\(^+\) infected cells. This indicates that the effect of viral infection on sodium conductance could not be mimicked simply by inhibiting protein synthesis.

**Other virion proteins**

It is possible that a virion component could, upon cell entry, affect sodium channels directly or indirectly to alter sodium conductance. To investigate the role of virion proteins in the electrophysiological changes during an infection, wt HSV 17\(^+\) was allowed to enter the DRG neurones but not to synthesise proteins. Any effects recorded would be due to virion components only. DRG neurones were treated with 50\(\mu\)g/ml cycloheximide for 30 minutes to inhibit host protein synthesis. The DRG neurones were then infected with wt HSV 17\(^+\) and incubated in the continuing presence of 50\(\mu\)g/ml cycloheximide for 24 hours. All wt HSV 17\(^+\) infected cells treated with cycloheximide showed sodium currents of normal amplitudes. The current traces shown in figure 4.3a exemplify recordings made in this set of experiments. Figure 4.3b shows that the normalised sodium current amplitude (nA/pF) of DRG neurones infected with wt HSV 17\(^+\) and in the presence of cycloheximide for 24 hours was not reduced compared to the normalised sodium current amplitude of wt HSV 17\(^+\) infected DRG neurones. This indicates that the virion proteins alone could not cause the loss of sodium conductance observed with a wt HSV 17\(^+\) infection for 24 hours.
Figure 4.3. The effect of protein synthesis inhibition on sodium current amplitude in wt HSV 17+ infected and uninfected DRG neurones.  a Sodium currents of DRG neurones treated with cycloheximide, and HSV infected with and without cycloheximide.  b The graph shows the mean normalised peak sodium current ± s.e.m from cells either, wt HSV 17+ infected, 50μg/ml cycloheximide treated or wt HSV 17+ infected in the presence of 50μg/ml cycloheximide. Normalised currents from DRG neurones treated with 50μg/ml cycloheximide for 24 hours and wt HSV 17+ infected cells in the presence of 50μg/ml cycloheximide for 24 hours were not significantly different, P>0.5, but were both significantly larger in size than the wt HSV 17+ infected (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison), P<0.0001.
As previously described, a family of sodium currents was evoked by test potentials of 5mV steps between -50mV and +25mV, after a hyperpolarising prepulse to remove sodium channel inactivation.

Figure 4.4 illustrates that the total sodium current-voltage relationships were not shifted during host neurone or viral protein synthesis inhibition compared to control or wt HSV 17+ infected neurones. A conductance-voltage curve was constructed for each recording and fitted with a Boltzmann function. The normalised mean conductance plotted against voltage is shown in figure 4.5. The $V_{50}$ and the slope factors were calculated from the best fit for each cell tested and the mean values are shown in table 4.1.

![Figure 4.4. The effects of protein synthesis inhibition on the sodium current-voltage relationship of wt HSV 17+ infected and uninfected DRG neurones. The graph shows the normalised sodium currents ± s.e.m plotted against command potential from control (■) n=16, cycloheximide treated (□) n=11, wt HSV 17+ infected (○) n=10, and wt HSV 17+ infected and cycloheximide treated for 24 hours (●) n=33 DRG neurones.](image)
Figure 4.5. Conductance-voltage relationship for sodium channels of DRG neurones during infection with wt HSV 17+ and/or treatment with cycloheximide. The relationship between \( g/g_{\text{max}} \) and test potential was determined from the current-voltage relationships shown in figure 4.4 as described in the text. The graph shows the normalised conductance ± s.e.m from control (■) \( n=16 \), cycloheximide treated (□) \( n=11 \), wt HSV 17+ infected (○) \( n=10 \), and wt HSV 17+ infected and cycloheximide treated for 24 hours (●) \( n=33 \) DRG neurones.
Table 4.1. Effects of cycloheximide and/or wt HSV 17+ infection on the electrophysiological characteristics of sodium currents in DRG neurones. $V_{50}$ refers to the potential ± s.e.m at which sodium conductance ($g$) is half of the maximum value ($g_{max}$).

The slope factor of the normalised conductance-voltage relationship is a function of the voltage dependence of sodium conductances. $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between the data points of the conductance-voltage plots of each cell. There was no significant difference between the groups (analysis of variance followed by Tukey’s post hoc analysis, $P>0.9$).

There was no statistically significant difference in the electrophysiological characteristics of sodium currents of DRG neurones infected with wt HSV 17+ in the presence or absence of cycloheximide. These data indicate that entry of a virion component in the absence of protein synthesis does not alter sodium conductance as observed with a wt HSV 17+ infection for 24 hours.

The TTX-S and TTX-R sodium currents were separated electrophysiologically by evoking currents with depolarising pulses to -20mV for 10 msec from two holding potentials (-50 and -120mV). At -50mV TTX-S channels were inactivated and the resulting currents are the TTX-R component. The combined TTX-S and TTX-R currents were evoked by the depolarising step to -20mV after the prepulse to -120mV. The TTX-S component was obtained by subtracting the TTX-R component from the total current. The TTX-S and TTX-R sodium currents of wt HSV 17+ infected, wt HSV 17+ infected and cycloheximide treated, cycloheximide treated and control cells were compared.
The TTX-S normalised currents (nA/pF) of cycloheximide treated DRG neurones or wt HSV $17^+$ infected DRG neurones in the presence of cycloheximide were not reduced in amplitude compared to the currents in control neurones. In contrast, TTX-S currents after wt HSV $17^+$ infection for 24 hours were significantly reduced (figure 4.6).

![Graph showing normalised TTX-S sodium current amplitudes of wt HSV $17^+$ infected and uninfected DRG neurones treated with cycloheximide for 24 hours. The graph shows the mean normalised peak TTX-S sodium current ± s.e.m of DRG neurones either treated with 50µg/ml cycloheximide, wt HSV $17^+$ infected, or wt HSV $17^+$ infected and treated with 50µg/ml cycloheximide for 24 hours. The normalised TTX-S sodium currents of wt HSV $17^+$ infected neurones were significantly smaller than the control and cycloheximide treated neurones, using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.0001. The normalised currents of cycloheximide treated and uninfected DRG neurones were not significantly different from each other, Student’s t test, P>0.5.]

Figure 4.6. Normalised TTX-S sodium current amplitudes of wt HSV $17^+$ infected and uninfected DRG neurones treated with cycloheximide for 24 hours. The graph shows the mean normalised peak TTX-S sodium current ± s.e.m of DRG neurones either treated with 50µg/ml cycloheximide, wt HSV $17^+$ infected, or wt HSV $17^+$ infected and treated with 50µg/ml cycloheximide for 24 hours. The normalised TTX-S sodium currents of wt HSV $17^+$ infected neurones were significantly smaller than the control and cycloheximide treated neurones, using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.0001. The normalised currents of cycloheximide treated and uninfected DRG neurones were not significantly different from each other, Student’s t test, P>0.5.

The TTX-R normalised currents (nA/pF) from neurones in different treatment groups are shown in figure 4.7. Again cycloheximide treatment of uninfected or wt HSV $17^+$
infected DRG neurones did not reduce current amplitude whereas a markedly reduced current was seen in wt HSV 17+ infected DRG neurones. The TTX-R normalised sodium current amplitudes were also unaffected by protein synthesis inhibition of uninfected and wt HSV 17+ infected DRG neurones.

![Diagram showing normalised TTX-R sodium current amplitudes of wt HSV 17+ infected and uninfected DRG neurones treated with cycloheximide for 24 hours.](image)

**Figure 4.7. Normalised TTX-R sodium current amplitudes of wt HSV 17+ infected and uninfected DRG neurones treated with cycloheximide for 24 hours.** The graph shows the mean normalised peak TTX-R sodium current ± s.e.m of DRG neurones either treated with 50µg/ml cycloheximide, wt HSV 17+ infected, or wt HSV 17+ infected and treated with 50µg/ml cycloheximide for 24 hours. The normalised TTX-R sodium currents of wt HSV 17+ infected neurones were significantly smaller than the control and cycloheximide treated neurones, using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.0001. The normalised currents of cycloheximide treated and uninfected DRG neurones were not significantly different from each other, Student’s t test, P>0.6.

In summary, neither inhibition of DRG neurones protein synthesis nor entry of viral virion components into DRG neurones, in the absence of any host or viral protein synthesis mimicked the loss of TTX-S and TTX-R sodium current amplitudes observed with wt HSV 17+ infection of DRG neurones for 24 hours.
Viral immediate early genes

The results presented in the section entitled "virus entry" (page 116) show that viral entry into DRG neurones and the effect of tegument protein vhs are not sufficient to cause loss of sodium conductance. Viral protein synthesis could, therefore, be important and the first set of viral proteins to be synthesised are encoded by viral immediate early genes. After viral entry into the cell, the immediate early genes are expressed by the host cell (Mackem & Roizman, 1981). This section investigates the role of immediate early gene expression in the loss of sodium conductance observed with wt HSV 17\(^+\) infection.

The regulatory immediate early genes code for ICP0, ICP4, and ICP27. Peak synthesis of immediate early proteins occurs between 2 and 4 hours post infection yet the proteins continue to accumulate nonuniformly throughout infection (Ackermann et al. 1984; Honess & Roizman, 1974). HSV mutants lacking ICP0, ICP4 and ICP27 were used to investigate the effect of regulatory immediate early genes in HSV infected DRG neurones.

Two different HSV ICP4 mutants were used. ICP4 (1), designated KOS 8117/43, had β-galactosidase inserted into ICP4 locus of both inverted repeats, as well as a deletion in the LATs (latency associated transcripts) (Dobson et al. 1990). This was constructed from mutation of parent strain KOS(M) with a deletion which abolished expression of the LATs (termed KOS 8117). A further interruption of immediate early gene encoding ICP4 then created KOS 8117/43 (Izumi et al. 1989). Thus, KOS 8117/43 could not express LATs or ICP4.

ICP4 (2), designated D30EBA, contains large deletions in the ICP4 gene coding sequences (Paterson & Everett, 1990). This mutant was constructed from the wild type Glasgow strain 17\(^+\). Infections with D30EBA were useful for direct comparison with wt HSV 17\(^+\) infected DRG neurones as both viruses share the same backbone.
The ICP27 deletion mutant used in these experiments was designated ZΔMN+ (Howard et al. 1997). The parental virus wt HSV 17+ had the entire ICP27 gene replaced by the Escherichia coli Lac Z gene under the strong promoter MMLV LTR (Maloney murine leukemia virus long terminal repeat promoter).

The mutant virus, dl1403, which lacks functional ICP0, was derived from the parental virus wt HSV 17+. Cell line M110, containing sequences encoding wild type ICP0 was used to grow the mutant virus. M110 cells complemented the growth of any progeny that either carried a lethal, or slow growing mutant. The mutant was made by homologous recombination with a plasmid containing the gene encoding ICP0 with 2Kb deletions. Progeny virus containing deletions in both copies of the genes encoding ICP0 were identified by Southern analysis, (Stow & Stow, 1986). The mutant phenotype, which reduced the yield of infectious virus by about 100 fold, could be rescued by restoring wildtype genes encoding ICP0.

Sodium currents were evoked by the one step depolarisation protocol and the sodium current amplitudes were normalised for cell size (current amplitude/cell capacitance nA/pF). Figure 4.8 shows the normalised sodium current amplitudes for cells infected for 24 hours with HSV 17+ mutants lacking functional immediate early genes ICP4, ICP27, and ICP0 together with the data from control and wt HSV 17+ infected DRG neurones. These results show that preventing a lytic infection with non functional ICP4 or ICP27 also prevents the loss of sodium current observed with wt HSV 17+ infection. Similarly, infection of DRG neurones with mutants lacking functional ICP0, which show inefficient onset of lytic infection, did not elicit the loss of sodium conductance seen with wt HSV 17+ infection.
Figure 4.8. The normalised total sodium current amplitude of DRG neurones infected with HSV lacking functional immediate early genes ICP4, ICP27 and ICP0. The graph shows the mean normalised peak sodium current ± s.e.m of DRG neurones either infected with wt HSV 17+, or HSV lacking functional immediate early genes ICP4, ICP27 and ICP0, or uninfected. Sodium currents of control or HSV lacking functional immediate early genes ICP4, ICP27 and ICP0 infected DRG neurones were all significantly larger than the sodium currents of wt HSV 17+ infected neurones P<0.005, but not significantly different from each other, P>0.6 (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison).

A family of sodium current activation curves were evoked as described previously. The normalised current-voltage relationships of DRG neurones either infected with wt HSV 17+, or HSV lacking functional immediate early genes ICP4, ICP27 and ICP0 are plotted in figure 4.9. The activation curve for the HSV lacking functional ICP0 showed a hyperpolarising shift which could be a reflection of the small sample size and the different properties of the TTX-S and TTX-R sodium currents. These results indicate that events downstream of immediate early gene expression result in the pathogenic loss of sodium conductance.
Figure 4.9. The sodium current-voltage relationship of DRG neurones infected with HSV lacking immediate early genes. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (○) n=10, HSV lacking functional ICP4 (1) ■ n=12, HSV lacking ICP4 (2) ● n=23, HSV lacking functional ICP27 (●) n=24 and HSV lacking functional ICP0 (□) n=8 infected DRG neurones.

Individual activation curves were also constructed from each neurone tested. The normalised conductance-voltage relationships derived from each current-voltage relationship are shown in figure 4.10. Statistical analysis of the electrophysiological values taken from the activation curve in figure 4.10 are displayed in table 4.2.
Figure 4.10. Conductance-voltage relationship for sodium channels of DRG neurones after 24 hours infection with HSV lacking immediate early genes. The relationship between \( g/g_{\text{max}} \) and test potential was determined from the current-voltage relationship shown in figure 4.9 as described in the text. The graph shows the normalised conductance \( ± \text{s.e.m} \) from control neurones (○) \( n=10 \), HSV lacking functional ICP4 (1) (■) \( n=12 \), HSV lacking ICP4 (2) (◆) \( n=23 \), HSV lacking functional ICP27 (●) \( n=24 \), and HSV lacking functional ICP0 (□) \( n=8 \) infected DRG neurones.
Table 4.2. Electrophysiological characteristics of DRG neurones infected with HSV lacking functional immediate early genes. $V_{50}$ refers to the potential ± s.e.m at which sodium conductance ($g$) is half of the maximum value ($g_{max}$). The slope factor of the normalised conductance-voltage relationship is a function of the voltage dependence of sodium conductances. $V_{50}$ and slope factors were calculated from Blotzmann curves fitted between the data points of the conductance-voltage plots of each cell. There was no significant difference between $V_{50}$ and slope factor values $P>0.6$.

Infection of DRG neurones with virus lacking functional regulatory immediate early genes did not significantly alter the voltage dependence of the sodium currents generated.

The total sodium currents evoked comprise TTX-S and TTX-R components. The amplitudes of TTX-R sodium currents tend to be smaller than those of the TTX-S currents. For this reason loss of TTX-R currents may go unnoticed in an analysis of total sodium currents and it was not clear whether the TTX-S and TTX-R sodium currents were both unaffected by infection with HSV mutants lacking the functional immediate early genes. For further clarification, the TTX-S and TTX-R sodium currents were separated on the basis of their inactivation properties. Briefly, the total sodium current was evoked by a 10 msec depolarising command to -20mV from a prepulse of -120mV. TTX-R sodium current was evoked from a prepulse of -50mV.

<table>
<thead>
<tr>
<th></th>
<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>-21.3 ± 1.1</td>
<td>5.1 ± 0.3</td>
<td>10</td>
</tr>
<tr>
<td>ICP4 (1)</td>
<td>-16.0 ± 2.7</td>
<td>6.5 ± 0.8</td>
<td>12</td>
</tr>
<tr>
<td>ICP4 (2)</td>
<td>-21.2 ± 1.3</td>
<td>5.0 ± 0.6</td>
<td>23</td>
</tr>
<tr>
<td>ICP27</td>
<td>-21.3 ± 1.8</td>
<td>6.0 ± 0.6</td>
<td>24</td>
</tr>
<tr>
<td>ICP0</td>
<td>-28.3 ± 0.9</td>
<td>3.4 ± 0.3</td>
<td>8</td>
</tr>
</tbody>
</table>
with a 10 msec command potential to -20mV. TTX-S sodium current was obtained by subtraction.

Figures 4.11 and 4.12 show the normalised TTX-S and TTX-R sodium currents (nA/pF) from DRG neurones infected with HSV mutants lacking functional immediate early genes. Although there is variability in the averaged data, there are no significant differences between the normalised mean TTX-S and TTX-R current amplitudes of DRG neurones infected with HSV mutants lacking the functional immediate early genes and control TTX-S and TTX-R sodium currents.

Figure 4.11. The TTX-S normalised mean sodium currents of DRG neurones infected with HSV lacking functional immediate early genes ICP4, ICP27 and ICP0. The graph shows the mean normalised TTX-S sodium current ± s.e.m of control DRG neurones or infected with HSV lacking functional immediate early genes ICP4, ICP27 and ICP0. There was no significant difference between the normalised current amplitudes (analysis of variance followed by Tukey’s post hoc analysis P>0.7).
Figure 4.12. The TTX-R normalised mean sodium currents of DRG neurones infected with HSV lacking functional immediate early genes ICP4, ICP27 and ICP0. The graph shows the mean normalised TTX-R sodium current ± s.e.m of control DRG neurones or infected with HSV lacking functional immediate early genes ICP4, ICP27 and ICP0. There was no significant difference between the normalised current amplitudes (analysis of variance followed by Tukey’s post hoc analysis P>0.4).

Although there is variability in the averaged data, especially the mean normalised TTX-R current evoked from DRG neurones infected with HSV mutants lacking ICP0, there was no significant difference in mean normalised TTX-R current amplitudes.

To investigate further the effects of infection with HSV mutants lacking functional immediate early genes, current-voltage relationships of TTX-S and TTX-R sodium currents were examined. A family of sodium currents was generated, after a prepulse to -120mV for 200 msec, with 10 msec command potentials between -80mV and +40mV. TTX-R sodium currents were generated after a prepulse to -50mV for 200 msec, with 10 msec command potentials between -80mV and +40mV. The TTX-S currents were obtained by subtraction of TTX-R currents from the total currents generated at each test potential.
The normalised current-voltage relationship of TTX-S currents from DRG neurones infected with HSV mutants lacking immediate early genes are shown in figure 4.13. There was no obvious difference in the activation curves of DRG neurones infected with HSV mutants lacking immediate early genes.

Figure 4.13. The TTX-S sodium current-voltage relationship of DRG neurons infected with HSV based vectors. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (○) n=10, and HSV lacking functional ICP4 (■) n=8, HSV lacking functional ICP27(●) n=6, HSV lacking functional ICP0 (□) n=4 infected DRG neurones.

Individual conductance-voltage relationships were derived from each family of TTX-S sodium currents obtained. The averaged normalised conductance-voltage relationships are shown in figure 4.14.
Figure 4.14. Conductance-voltage relationship for TTX-S sodium channels after infection with HSV without functional immediate early genes for 24 hours. The graph shows mean normalised conductance ± s.e.m from control neurones (○) n=10, HSV lacking functional ICP4 (■) n=8, HSV lacking functional ICP27 (●) n=6, HSV lacking functional ICP0 (□) n=4. The continuous lines were obtained by fitting a Boltzmann function to the mean normalised data.

Table 4.3 displays the averaged $V_{50}$ and slope factor values calculated by fitting a Boltzmann function to the individual TTX-S conductance-voltage relationships for each neurone.

Current-voltage relationships were also constructed for TTX-R currents and the individual current-voltage relationships were normalised with respect to peak current. The averaged data are shown in figure 4.15.
Figure 4.15. The TTX-R sodium current-voltage relationship of DRG neurons infected with HSV based vectors. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (O) n=10, and HSV lacking functional ICP4 (■) n=8, HSV lacking functional ICP27 (●) n=6, HSV lacking functional ICP0 (□) n=4 infected DRG neurones.

Analysis of the current-voltage relationship of the TTX-R currents indicates that there was no obvious shift in the voltage dependence when infected with the immediate early gene wt HSV 17^ mutants. From each current-voltage relationship, the conductance-voltage relationships were calculated and normalised to peak conductance (g/gmax). The averaged normalised data are presented in figure 4.16.
Figure 4.16. Conductance-voltage relationship for TTX-R sodium channels after infection with HSV without functional immediate early genes for 24 hours. The graph shows mean normalised conductance ± s.e.m from control neurones (O) n=10, HSV lacking functional ICP4 (■) n=8, HSV lacking functional ICP27 ( ●) n=6, HSV lacking functional ICP0 (□) n=4. The continuous lines were obtained by fitting a Boltzmann function to the mean normalised data.

V<sub>50</sub> and slope factor values were calculated by fitting a Boltzmann function to each individual activation curve and the mean values are shown in table 4.3. The results shown in table 4.3 demonstrate that the activation characteristics of TTX-S and TTX-R currents are not affected by infection with wt HSV 17<sup>+</sup> lacking functional immediate early genes.
Table 4.3. Electrophysiological characteristics of DRG neurones infected with HSV lacking functional immediate early genes. The $V_{50}$ from the TTX-S conductance-voltage relationship is significantly larger than the $V_{50}$ from the TTX-R conductance-voltage relationship in each case. The TTX-R and TTX-S slope factors and $V_{50}$ values from cells infected with HSV lacking functional immediate early genes do not differ significantly from the control values (analysis of variance followed by Tukey's post hoc analysis $P>0.7$). $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between the data points of the conductance-voltage plots of each cell.

In summary, infection of DRG neurones with HSV mutants lacking functional ICP4, ICP27 or ICP0 had no significant effect on the amplitudes or activation characteristics of the TTX-S and TTX-R sodium currents. These data show that the immediate early genes are necessary for the loss of sodium conductance observed with wt HSV 17$^+$.

Viral DNA replication

The experiments described in the previous sections have elucidated that virus entry into the host cell and virus host shut off protein is not sufficient to cause the loss of sodium conductance observed with a wt HSV 17$^+$ infection, and that an event downstream of regulatory immediate early gene expression is necessary for the loss of sodium conductance. The next stage in the lytic cycle of replication is the expression
of the early genes followed by DNA replication. This section investigates the effects of preventing viral DNA replication on loss of sodium currents.

Acyclovir is the generic name for 9-(2-hydroxy-ethoxymethyl)guanine, which has antiviral selectivity based on phosphorylation of the drug to form acyclovir monophosphate by viral enzyme thymidine kinase but not by cellular enzymes (Elion et al. 1977). Acyclovir monophosphate is converted by host cell enzymes to form acyclovir triphosphate. This interferes with viral DNA synthesis by inhibiting viral DNA polymerase as well as acting as a chain terminator when incorporated into a growing DNA strand (Elion, 1983). The late genes can be subdivided on the basis of their dependence on viral DNA replication for expression. The "leaky" late genes are expressed at low levels in the absence of DNA replication, whilst the true late genes stringently require viral DNA synthesis (Holland et al. 1980; Costa et al. 1985). Since expression of true late genes is dependent on viral DNA replication, wt HSV 17+ infected cells treated with acyclovir would be expected to express immediate early, early and "leaky" late genes but not true late genes (Silver & Roizman, 1985). In this experiment, the dependence of sodium current loss on viral DNA synthesis was investigated by infecting DRG neurones with wt HSV 17+ in the presence of 50μM acyclovir.

Sodium currents were generated with the one step depolarisation protocol (figure 4.17a). Figure 4.17b shows the normalised sodium current amplitudes (nA/pF) of acyclovir treated, acyclovir treated and wt HSV 17+ infected DRG neurones compared with uninfected, control DRG neurones and wt HSV 17+ infected DRG neurones. The sodium amplitudes of the total sodium currents in neurones from both acyclovir treated and wt HSV 17+ infected, acyclovir treated cultures were not significantly different from the amplitudes found in control (uninfected) neurones. In contrast, the normalised mean sodium current was significantly reduced in wt HSV 17+ infected DRG neurones. The data illustrate that when viral DNA replication was inhibited and the true late genes were not expressed, the sodium currents were not lost as with infected neurones in the absence of acyclovir.
Figure 4.17. The effect of viral DNA synthesis inhibition on normalised sodium current amplitude of wt HSV 17+ infected and uninfected DRG neurones. The graph shows the mean normalised peak sodium current ± s.e.m of control DRG neurones or, wt HSV 17+ infected, 50µM acyclovir treated, or wt HSV 17+ infected in the presence of 50µM acyclovir. The normalised sodium current from wt HSV 17+ infected cells was significantly smaller than normalised sodium currents recorded from 50µM acyclovir treated, wt HSV 17+ in the presence of 50µM acyclovir and control neurones, **P<0.0001, using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison.
Furthermore, addition of 50μM acyclovir to control or wt HSV 17+ infected DRG neurones did not affect the sodium current-voltage relationship, as shown by the mean normalised currents (figure 4.17) and the mean normalised current-voltage plots (figure 4.18).

Figure 4.18. The effects of acyclovir on the sodium current-voltage relationship of wt HSV 17+ infected and control DRG neurones. The graph shows the normalised sodium currents ± s.e.m plotted against command potential from control (●) n=11, wt HSV 17+ infected (□) n=3, acyclovir treated for 24 hours (○) n=6, and wt HSV 17+ infected and acyclovir treated for 24 hours (■) n=11 DRG neurones.
Figure 4.19. Conductance-voltage relationships for sodium channels during infection with wt HSV 17£ and/or treatment with acyclovir. The relationship between g/gmax and test potential was determined from the current-voltage relationships shown in figure 4.18 as described in the text. The graph shows normalised conductance ±s.e.m from control neurones (●) n=11, wt HSV 17£ infected neurones (□) n=3, acyclovir treated neurones for (○) n=6, and wt HSV 17£ infected neurones treated with acyclovir (■) n=11. The continuous lines were obtained by fitting the normalised data to a Boltzmann function.

Conductance-voltage relationships for the total sodium current were determined for each neurone and fitted with a Boltzmann function (figure 4.19). The mean $V_{50}$ and slope factor values from this analysis are shown in table 4.4. There was no significant difference in the $V_{50}$ or slope factors of the conductance-voltage relationships of control or wt HSV 17£ infected DRG neurones treated with 50µM acyclovir (analysis of variance followed by Tukey’s post hoc analysis P>0.2).
Table 4.4. Effects of acyclovir and/or wt HSV 17\(^+\) infection on the electrophysiological characteristics of sodium currents in DRG neurones. \(V_{50}\) refers to the potential ± s.e.m at which sodium conductance (g) is half of the maximum value (g\text{max}). The slope factor of the normalised conductance-voltage relationship is a function of the voltage dependence of sodium conductances. \(V_{50}\) and slope factors were calculated from Boltzmann curves fitted between the data points of the conductance-voltage plots of each cell.

The TTX-S and TTX-R sodium currents of acyclovir treated control and wt HSV 17\(^+\) infected DRG neurones were separated electrophysiologically in order to examine the effect of acyclovir on sodium channels subtypes. Figure 4.20 and figure 4.21 demonstrates that without the expression of true late genes TTX-S and TTX-R sodium currents respectively are not reduced upon acyclovir treatment or wt HSV 17\(^+\) infection in the presence of acyclovir (P>0.1). In contrast, infection with wt HSV 17\(^+\) results in a statistically significant decrease in sodium current amplitude (P<0.0005).
Figure 4.20. The effect of acyclovir on normalised TTX-S sodium current amplitude of wt HSV 17\(^+\) infected and uninfected DRG neurones. The graph shows the mean normalised peak TTX-S sodium current ± s.e.m of DRG neurones, treated with 50µM acyclovir, wt HSV 17\(^+\) infected, or wt HSV 17\(^+\) infected and treated with 50µM acyclovir. The TTX-S normalised sodium currents of wt HSV 17\(^+\) infected cells were significantly smaller than the currents of the acyclovir treated cells (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, \(P<0.0005\)).
Figure 4.21. The effect of acyclovir on normalised TTX-R sodium current amplitude of wt HSV 17+ infected and uninfected DRG neurones. The graph shows the mean normalised peak TTX-R sodium current ± s.e.m of DRG neurones, treated with 50μM acyclovir, wt HSV 17+ infected, or wt HSV 17+ infected and treated with 50μM acyclovir. The TTX-R normalised sodium currents of wt HSV 17+ infected cells were significantly smaller than the currents of the acyclovir treated cells (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.0005).

From these results, it is possible to speculate that events or proteins synthesised downstream of viral DNA synthesis are required for the observed loss of sodium conductance.
Cosmids

In a further attempt to narrow down which part of the viral genome was responsible for causing the sodium current loss during a wt HSV 17\(^+\) infection a cosmid library was employed (Cunningham & Davison, 1993). The cosmid library consists of five cosmids containing large wt HSV 17\(^+\) DNA fragments, which overlap and comprise the entire wt HSV 17\(^+\) genome. Previously all five cosmids have been transfected into BHK cells and have produced viral plaques (Cunningham & Davison, 1993), thus indicating that a fully productive infection is possible with cosmid DNA, although the production of plaques was 24 hours later than with transfection of wt HSV 17\(^+\) DNA. The aim of the experiment was to microinject all of the five wt HSV 17\(^+\) DNA fragments followed by electrophysiological recording in the voltage-clamp mode after 24 hours. Expression of the genes on the five fragments should result in a lytic infection and may cause loss of sodium conductance. Subsequent microinjection of a combination of fragments followed by electrophysiological recordings may show that one of the fragments contains gene(s) encoding protein(s) involved in loss of sodium conductance.

The five cosmids were grown and purified from bacterial cultures. The cosmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Restriction digests were performed on the purified cosmid DNA, to ensure that the correct DNA was harvested. The restriction digests showing the correct restriction fragment patterns are shown in figure 4.22.
Figure 4.22. The restriction digest of cosmids 6, 14, 28, 48 and 56. The cosmids 6, 14, 28, 48 and 56 which together contain the entire wt HSV 17+ genome, were each cut with the restriction enzyme Kpn1. The DNA fragments were separated by agarose gel electrophoresis. The agarose gel contained ethidium bromide and the fragments were visualised by u.v. irradiation. Lane 1 shows the 1 Kb ladder, lane 2 shows uncut cosmid 6 DNA, lane 3 shows the restriction pattern of cosmid 6, lane 4 shows the restriction pattern of cosmid 14, lane 5 shows the restriction pattern of cosmid 28, lane 6 shows the restriction pattern of cosmid 48, and lane 7 shows the restriction pattern of cosmid 56. The restriction patterns of the cosmid DNA from bacterial cultures, purified by equilibrium centrifugation in CsCl-ethidium bromide gradients were as expected.

The cosmids were microinjected along with FITC (fluorescein isothio-cyanate) as a fluorescent marker which is retained in viable cells. Alternatively, cosmids were coinjected with a plasmid pEGFP-C1 encoding GFP (green fluorescent protein). The presence of fluorescent cells show both that the cells are viable and that the DNA can be transcribed in those cells.

Figure 4.23a shows DRG neurones 48 hours after microinjection with a FITC marker and the wt HSV 17+ cosmid library. After 48 hours the cell still fluorescence indicating that it is still viable. Figure 4.23b shows a DRG neurone which was
microinjected with the wt HSV 17^ cosmid library and a GFP plasmid after incubation for 24 hours.

**Figure 4.23.** DRG neurones containing either FITC or GFP.  

**a** Cosmid DNA and FITC were microinjected into a DRG neurone left in culture for 48 hours.  

**b** A DRG neurone was microinjected with cosmid DNA and a plasmid containing a GFP gene and incubated for 24 hours in F14 culture medium.

The whole cosmid library was microinjected into DRG neurones with a FITC or a GFP marker. Sodium currents of fluorescent, microinjected DRG neurones were evoked with a step depolarisation from a holding potential of -80mV to +10mV for 13msec. The sodium currents generated were normalised for cell size (nA/pF) the mean data are shown in figure 4.24.
**Figure 4.24. The effect of microinjecting wt HSV 17+ cosmid library into DRG neurones.** DRG neurones were microinjected with the wt HSV 17+ cosmid library plus the a FITC or GFP marker. The neurones were then incubated between 24-48 hours. The graph shows the mean normalised sodium current ± s.e.m. There was no significant difference between the groups tested (analysis of variance followed by Tukey's post hoc analysis $P > 0.7$ although the n values are rather low for concluding non significance).

All neurones microinjected with the cosmid library had a sodium current. The currents were not significantly different from those generated from cells injected with marker only. Cells tested at 24 and 48 hours did not differ significantly in their normalised current amplitude so were grouped together for analysis. Microinjected neurones did not tend to produce neurites and space clamp problems were not experienced. The number of viable neurones surviving the microinjection was relatively low, i.e. 10% success rate. It was not practically feasible to use a HSV protein antibody for immunohistochemistry to visualise the presence of viral proteins in microinjected cells since there were too few cells and so there is no direct evidence that any of the cosmid genes were expressed in DRG neurones. The failure of the cosmids to alter sodium currents in these preliminary experiments precluded further planned experiments in this series.
Discussion

The wt HSV 17\(^+\) replication cycle has many stages (for review see chapter 1), which can be isolated using pharmacological agents. The data in this chapter illustrates the effects that different stages in the replication cycle had on DRG neurone sodium currents. Discovering which part of the replication cycle is involved in the loss of sodium conductance is important for elucidation of a possible mechanism of action.

Infecting DRG neurones in the presence of cycloheximide allows virion particles to enter the cell, without subsequent viral protein synthesis and hence viral replication. Under these conditions the loss of sodium currents seen with wt HSV 17\(^+\) infection was not observed. This finding was consistent with the time of onset of sodium current loss described in chapter 3. The onset of sodium conductance loss would be expected to be earlier than 24 hours if viral entry was sufficient to cause the loss. It is also worth noting that expression of viral immediate genes does not require host or viral protein synthesis (Honess & Roizman, 1974), as in the presence of cycloheximide, the immediate early genes will still be expressed but not translated (Fenwick & Clark, 1983). The high levels of viral immediate early gene mRNA present in cycloheximide treated neurones, did not affect sodium conductance.

One particular virion tegument component which enters DRG neurones upon infection is the virion host shutoff protein (vhs), which inhibits host protein synthesis (Fenwick & Clark, 1982). Very few host proteins are translated during a wt HSV 17\(^+\) infection and it is possible that the loss of host cell protein synthesis could be the cause of sodium conductance loss. This seems unlikely to account for the loss of sodium channel conductance, because previous studies have shown sodium channels turnover in rat brain neurones with a half life of 72 hours (Schmidt & Catterall, 1986). An intracellular pool of newly synthesised sodium channel \(\alpha\) subunits, was the rate limiting step in the regulation of sodium channel density on neurone surface. The fact that neurones contain an intracellular pool of \(\alpha\) subunits and that sodium channels have a slow turnover, suggest that it is unlikely that sodium channels themselves are
lost from the cell membrane as a result of protein synthesis inhibition. It is possible, however, that another protein essential for sodium channel function with a higher turnover and with no intracellular pool to replenish loss, is depleted during protein synthesis inhibition. Examples of such proteins could include syntrophin, dystrophin, or actinin which have a role in clustering and localisation of ion channels. Another possibility is that the virion may de-repress the synthesis of a host inhibitor of sodium channels. A possible example of this can be found with the transcription factor REST (RE1-silencing transcription factor), which silences the cloned type II sodium channel promoter in non neuronal cell types (Chong et al. 1995). The presence of the repressor element 1 (RE1) in the sodium channel regulatory region, where REST binds, silences the sodium channel gene. Non-neuronal cells contain high levels of REST, whilst neuronal cells contain very little. It is possible that wt HSV 17+ infected DRG neurones have elevated levels of REST and thus inhibit the transcription of sodium channels. This situation, however, would not result in such a dramatic loss of sodium channels after 24 hours, as the half life of functional sodium channels is 72 hours.

After the virus has entered the DRG neurones and inhibited host cell protein synthesis, the first set of genes in the temporal cascade, the immediate early genes are expressed. The role of the immediate early genes in the loss of sodium currents was investigated by infecting DRG neurones with wt HSV 17+ mutants lacking functional immediate early genes ICP4, ICP27 and ICP0. ICP4, and ICP27 have regulatory functions and are both essential for the replication of wt HSV 17+ (Sacks et al. 1985; Watson et al. 1979). An infection with mutants lacking functional ICP4, results in an overexpression of the remaining immediate early genes. Thus overexpression of immediate early genes ICP0, ICP22, ICP27 and ICP47, did not affect the sodium conductance. It is possible to speculate from these results that ICP4 is the protein responsible for the loss of sodium conductance. This possibility can be eliminated by the finding that sodium channel loss did not occur after infection with mutants lacking functional ICP27, which results in an overexpression of the remaining immediate early genes. The data show that overexpression of immediate early genes ICP0, ICP22, ICP4 and ICP47 did not affect the sodium conductance. The data shown here
demonstrated that replication defective virus was incapable of altering sodium channel conductance.

Mutants lacking functional ICP0 are still able to replicate although at a more sluggish rate (Stow & Stow, 1986). DRG neurones infected with 5p.f.u/neurone of HSV mutant lacking ICP0 for 24 hours, all still had sodium currents. Neurones were not tested at times subsequent to 24 hours as they produced neurites and were too fragile to replate. It is possible that after a longer period of time, sufficient for viral replication, sodium currents may have been lost. It could also be argued that the multiplicity of infection used was insufficient to result in lytic viral growth. It has been reported in the literature that increasing the multiplicity of infection will result in a full lytic cycle. It is unfortunate in this instance that the cell lines were not available to grow a stock of ICP0 and there was insufficient available to infect DRG neurone cultures at a higher multiplicity of infection. It is possible then, that infection with an ICP0 mutant virus resulting in lytic growth, may have resulted in loss of sodium conductance, but this remains only a speculation.

The simplest conclusion from these results is that the mechanism causing loss of sodium conductance occurs downstream of immediate early gene expression. After immediate early gene expression the early genes, encoding proteins mostly involved with DNA replication machinery, are expressed. This is followed by viral DNA replication. When viral DNA replication is inhibited using acyclovir, true late genes are not expressed. Expression of true late genes negatively regulates the expression of the early genes. Preventing the expression of true late genes with acyclovir results in a build up of early proteins in the cell. The experiments exploring the role of DNA replication in the occurrence of electrophysiological changes, have been described in this chapter. DNA replication was necessary for the occurrence of sodium current loss. This can be interpreted as showing that an over abundance of early proteins cannot cause the effect and that DNA replication and the expression of true late proteins is necessary to cause the loss of sodium currents. Since there is only low level expression of "leaky late" genes in the absence of DNA replication, it is possible that higher amounts of "leaky late" proteins are required for the electrophysiological changes. It is tempting to conclude that a single true late protein is responsible for the
electrophysiological changes. Although this is possible, it is also legitimate to suggest that a process occurring after DNA replication, which may be attributed to the function of many proteins, causes the changes in neurone excitability. The process of DNA replication itself could potentially alter the state of the neurone and indirectly result in the loss of excitability.

Following the line of reasoning which suggests that a single gene product causes the decreased excitability, an attempt was made to narrow down the causal gene. A cosmid library containing five overlapping fragments of the entire wt HSV 17\(^+\) genome was microinjected into DRG neurones. Cunningham & Davison (1993), observed that transfection of the complete cosmid library would lead to a productive infection in BHK cells. The microinjected DRG neurones were analysed for changes in sodium current amplitudes. A lytic infection in the neurones was predicted. Successfully microinjected cells were visualised with FITC or GFP. Recordings were made from these cells at 24 and 48 hours following microinjection. There were no changes in the sodium currents of the microinjected cells. As all five cosmids microinjected together did not cause the changes in the sodium conductance, these experiments could be taken no further.

Some of the possible reasons why the cosmids were unable to cause the effects of the wt HSV 17\(^+\) infection are described below. One possibility is that the genes on the cosmid DNA were not expressed in DRG neurones. This idea is based on the fact that there are high levels of the transcription factors Oct-2.4 and Oct-2.5 in sensory neurones. When a HSV virion enters a cell the tegument transactivator ICP25 forms a complex with transcription factor Oct-1, which initiates the transcription of viral immediate early genes. In sensory neurones, however, the transcription factors Oct-2.4 or Oct-2.5 are abundant and they inhibit the binding of the ICP25-Oct-1 transactivator complex to the viral immediate early gene promoters and thus prevent the high level activity of HSV immediate early gene promoters required for a lytic infection. It is for this reason that neuronal forms of the transcription factor Oct-2 are thought to play a role in the establishment of latency (Lillycrop et al. 1993). Lytic infections occur in sensory neurones when there is sufficient ICP25-Oct-1 complex to
overcome the Oct-2.4 or Oct-2.5 viral immediate early gene promoter block. As the
wt HSV 17" genome is microinjected naked and not as part as a virion, the virion
protein ICP25 (Vmw65) is not present and so the ICP25-Oct-l transactivating
complex is not formed. Thus the lack of ICP25 could prevent sufficient expression of
viral immediate early genes to initiate a lytic infection. If the five cosmids injected
together were incapable of producing a lytic infection then loss of sodium conductance
would not be expected.

It is possible that the markers used to visualise successfully microinjected cells
interfered in some way with the expression of the cosmids. Two separate marker
systems were used in the hope that at least one of them would not interfere with
cosmid gene expression. The plasmid containing the gene for GFP protein was
coinjected with the cosmid library. It is possible that the strong promoter (CMV IE)
on the plasmid containing the gene encoding GFP, competes with the cosmid
promoters and inhibits their expression. FITC injection should not affect gene
expression unless it produces free radicals upon exposure to light. This problem was
avoided by keeping the cells darkened except immediately prior to recording. Overall,
it is unlikely that the markers affected the expression of cosmid genes.

It is also possible that as the genome was split into five segments the relevant gene
involved in modification of neuronal electrophysiology was rendered non functional.
The genome fragments are overlapping and there is clearly no perturbation of an
essential gene as productive infections were achieved with the cosmid library in BHK
cells (Cunningham & Davison, 1993).

If microinjection of the cosmid library had induced a loss of sodium conductance in
DRG neurones, the next step would have been either to microinject each cosmid
individually or in combinations with each other. There was the possibility that a gene
or genes, activated independently of other genes in the genome, may be singly
responsible for the loss of sodium conductance described. The microinjection of
cosmid(s) that did result in decreased sodium currents would have been analysed for
the presence of late genes. Plasmids containing each late gene found would be
obtained and microinjected into DRG neurones. The aim of such experiments would
be to isolate a single gene product that is directly responsible for altering sodium
currents. Since the microinjection of all five cosmids did not produce a lytic infection, these experiments were abandoned.

In summary, the results of the experiments described in this chapter show that for loss of sodium conductance virion entry alone was insufficient, whilst viral protein synthesis as well as DNA replication were necessary and that either a late protein or late process, i.e. down stream of viral DNA replication, was required.
Chapter 5

Mechanism of sodium conductance loss

Introduction

The previous chapters 3 and 4 have described that the TTX-S and TTX-R sodium currents of DRG neurones are selectively lost 24 hours after infection with wt HSV 17+. The mechanism of loss of sodium conductance can be attributed to an event downstream of viral DNA replication in the lytic cycle of wt HSV 17+. This event could be the synthesis of a late protein which, by an unknown mechanism, causes the loss of sodium currents. It is the unknown mechanism which is under scrutiny in this chapter.
One could speculate that the loss of sodium current was directly linked to a loss of sodium channels. Decreased sodium channel density from the surface of DRG neurones would certainly result in a reduction of sodium conductance. A possible mechanism for sodium channel loss from the plasma membrane of DRG neurones is down regulation by internalisation.

There is evidence in the literature that HSV-1 infected cells had increased internalisation of cell surface proteins. For example, the altered endocytosis of low density lipoprotein (LDL) receptors during wt HSV 17+ infection of arterial smooth muscle cells showed this characteristic. LDL receptors provide cells with an exogenous source of cholesterol for making new membrane. When arterial smooth muscle cell cultures were infected with wt HSV 17+ the intracellular levels of cholesterol and cholesteryl ester increased by more than 50 fold and showed an increased binding and internalisation of $[^{125}\text{I}]$-LDL (Hsu et al. 1995). If, however, the arterial cell cultures were infected with a HSV-1 viral protein kinase mutant, containing a deletion in gene U53, the increased cholesterol accumulation did not occur. This suggested a correlation between viral protein kinase activity and increased LDL internalisation and thus accumulation of cholesterol (Hsu et al. 1995; Purves et al. 1987). It is not clear, however, whether this correlation stems from viral kinase enhanced LDL receptor internalisation or by altered activity of kinase dependent proteins involved in cholesterol metabolism, such as, 3-hydroxy-3-methylgluaryl-coenzyme A (HMG-CoA) reductase which catalyses the rate limiting step in cholesterol synthesis, (Goldstein & Brown, 1990).

This evidence shows that certain cells infected with HSV-1 are capable of increased internalisation of surface membrane proteins and that this may be protein kinase dependent. More specifically of interest here is whether sodium channels are internalised in wt HSV 17+ infected DRG neurones.

Previously, it has been shown that incubation of neonatal rat brain cultures with veratridine (a sodium channel activator) in depolarising medium, such as 50mM KCl, for as short a period as one hour, results in a dramatic decrease in surface sodium channels (Dargent & Couraud, 1990). In these experiments the density of sodium
channels was monitored by $[^3]H$ saxitoxin or in some cases $[^{125}]I$ scorpion toxin binding, where scorpion toxin was both the channel activator and probe. Experiments to investigate if the decrease in surface sodium channels was a result of internalisation were based on the work of Haigler et al. (1980). In these experiments, radiolabeled ligands bind to cell surface receptors (in this case sodium channels), and after a period of incubation the cells were washed with a weak acid to remove ligand bound to the receptors on the cell surface. After the weak acid wash, any radioactivity associated with the cells would result from internalised probe bound to sodium channels. Acid resistant radioactivity was detected in neonatal rat brain cultures which had been treated with veratridine in depolarising medium, indicating that sodium channels were internalised. Synaptosomes prepared from adult rat brains or adult hippocampal slices did not behave similarly, as internalisation of sodium channels was not observed (Dargent et al. 1994).

The prolonged opening of sodium channels, either by treatment with veratridine or scorpion toxin, allows sodium influx and an increase in intracellular sodium concentration. The fact that acid resistant radioactivity is not present when experiments were performed in sodium free media or in the presence of sodium channel blocker TTX suggested that increased intracellular sodium concentration may be the trigger for sodium channel internalisation (Paillart et al. 1996). The sodium ionophore amphotericin B also induces sodium channel down regulation (Dargent & Couraud, 1990). The experiments in neonatal rat brain cultures provide evidence of a mechanism for neuronal voltage-gated sodium channel internalisation, triggered by an increase in intracellular sodium concentration, resulting in a loss of sodium channels from the cell surface and hence a loss of sodium conductance (Dargent et al. 1994). Internalisation of sodium channels after sodium influx was not mediated by protein kinase C, cAMP dependent protein kinase, cGMP protein kinase, tyrosine phosphorolation or intracellular calcium concentration (Dargent et al. 1995).

Sodium conductance is lost during a wt HSV 17+ infection of DRG neurones. The experiments described in this chapter investigate the possibility that the loss of sodium conductance observed with wt HSV 17+ infection were the result of sodium channel internalisation. One explanation could be that during a wt HSV 17+ infection
intracellular sodium concentration rises, triggering a down regulation of sodium channels by internalisation. The effects of increased intracellular sodium concentration in DRG neurones has not been analysed before. The possibility that sodium channels are internalised during a wt HSV 17+ infection, and the possibility that any such internalisation is triggered by raised intracellular sodium concentration, was explored.

Results

Inhibitors of internalisation

The membrane internalisation inhibitors, chloroquine, ammonium chloride and bafilomycin A₁ were used to investigate the effect of inhibiting internalisation during a wt HSV 17+ infection of DRG neurones. Chloroquine and ammonium chloride are lipophilic, acidotropic weak bases and in their unprotonated form enter cells and endosomes readily. Upon entering an acidic environment they become protonated and too polar to pass back through the endosome membrane. The fast change (minutes) in endosomal pH is due to neutralisation of protons by the weak base. Bafilomycin A₁ specifically inhibits vacuolar type H⁺ ATPases (V-ATPase) which are responsible for the acidification of vacuolar compartments such as clatherin coated pits, endosomes and lysosomes. Inhibition of the V-ATPases results in neutralisation of acid endosomes. The change in pH inhibits endosomal enzymes which inhibits internalisation by preventing the dynamic cycling between endosomes and the plasma membrane.

It is worth noting that wt HSV 17+ does not depend on endocytosis and acid pH for their penetration in contrast to Influenza virus and Semliki Forest virus (White et al. 1981; Morgan et al. 1968). Inhibitors of endocytosis which neutralise acid endosomes should not affect wt HSV 17+ adsorption or penetration. Just in case, however, the internalisation inhibitors were added to the medium 1 hour after infection with wt HSV 17+ to allow time for viral adsorption and penetration.
To determine the effects of inhibiting internalisation on the sodium currents of DRG neurones infected with wt HSV 17+, 100μM chloroquine, 25mM ammonium chloride and 1μM bafilomycin A1 were separately added to the media of infected and uninfected DRG neurone cultures which were then incubated for a further 24 hours. Sodium currents were generated from a holding potential of -80mV by a step depolarisation to +10mV for 13 msec (this protocol will be referred to as the one step depolarisation protocol throughout this chapter). Representative sodium current traces are shown in figure 5.1a. Figure 5.1b shows the sodium current amplitudes normalised for cell size (nA/pF) of wt HSV 17+ infected DRG neurones and infected or uninfected DRG neurones treated with internalisation inhibitors. The normalised sodium current amplitudes of wt HSV 17+ infected DRG neurones were significantly smaller than those of infected or uninfected DRG neurones treated with internalisation inhibitors. Infected and uninfected DRG neurones treated with internalisation inhibitors did not vary significantly in normalised sodium current amplitude. Additionally, the wt HSV 17+ infected DRG neurone treated with the internalisation inhibitors all had sodium currents. This demonstrates that inhibitors of internalisation were able to prevent the loss of sodium conductance which was observed after wt HSV 17+ infection for 24 hours.
Figure 5.1. The effect of internalisation inhibitors on control and wt HSV 17+ infected DRG neurones. a Sodium current traces of infected and uninfected DRG neurones treated in the presence or absence of bafilomycin A1. b The mean normalised current amplitude ± s.e.m of control and wt HSV 17+ infected DRG neurones treated with inhibitors of internalisation, bafilomycin A1 (1µM), chloroquine (100µM), and ammonium chloride (25mM). The only significant difference was between control current amplitudes and wt HSV 17+ infected current amplitudes, P<0.0001. The current amplitudes of wt HSV 17+ infected DRG neurones were significantly different to the current amplitudes of DRG neurones treated with bafilomycin A1, chloroquine or ammonium chloride during wt HSV 17+ infection for 24 hours, P<0.0001. The normalised sodium current amplitudes of DRG neurones treated with internalisation inhibitors were not significantly different from each other, P>0.5. Statistical analysis was examined by the Kruskal-Wallis statistical test, followed by the Kolmogorov-Smirnov pairwise comparison.
The sodium current-voltage relationships were constructed to investigate the possibility that inhibitors of internalisation had an effect on the voltage dependence of the sodium currents which could not be identified by analysis of the currents generated after a depolarisation to a single voltage. Prior to evoking sodium currents a prepulse to -120mV was applied to remove sodium channel inactivation. The family of sodium currents was then evoked by a series of command potentials at 5mV intervals between -50mV and +25mV. The peak of each current evoked was plotted against command potential to construct a current-voltage relationship. The data were normalised with respect to the maximum current evoked for each neurone. The mean normalised current-voltage relationships are shown in figure 5.2 for uninfected DRG neurones treated with inhibitors of internalisation. Families of sodium currents described throughout this chapter were generated with the same voltage protocol described above. The sodium current-voltage relationships which appear within this chapter were also constructed as described above. The family of sodium currents were generated after a prepulse to -120mV to remove sodium channel inactivation. The current-voltage relationships show that a prepulse to -120mV did not reveal a current in neurones where no current had been seen with the one step depolarisation protocol.
Figure 5.2. The effect of treatment with internalisation inhibitors on the current-voltage relationship of DRG neurones. The graph shows the normalised current amplitudes ± s.e.m from control (●) n=5 DRG neurones, and bafilomycin A₁ (△) n=5, chloroquine (∇) n=5, and ammonium chloride (□) n=7 treated DRG neurones for 24 hours.

The activation curves were also constructed for each cell tested by normalising the conductance with respect to peak conductance (g/gmax). The mean normalised conductance-voltage relationships are shown in figure 5.3. DRG neurones incubated in the presence of ammonium chloride or bafilomycin A₁ show almost overlapping activation curves as reflected in the mean slope factors and $V_{50}$ values shown in table 5.1 (page 175). The mean activation curve of DRG neurones incubated in the presence of chloroquine appears to be slightly shifted from the others. The error bars, however, are large and the mean slope factors and $V_{50}$ values were not significantly different from the others, (see table 5.1, page 175). There was no shift in the activation curves sufficient to affect the normalised sodium amplitude at +10 mV. Thus incubating DRG neurones in the presence of internalisation inhibitors did not significantly alter the voltage dependence of sodium currents.
Figure 5.3. The effect of treatment with internalisation inhibitors on the conductance-voltage relationship of DRG neurones. The graph shows the normalised conductance ± s.e.m from control (●) n=5, and from DRG neurones whilst being treated with, bafilomycin A₁ (Δ) n=5, chloroquine (▽) n=5, and ammonium chloride (□) n=7. The continuous lines were obtained by fitting the mean normalised data to a Boltzmann function.

The normalised current-voltage relationships for wt HSV 17<sup>+</sup> infected DRG neurones treated with inhibitors of internalisation were calculated as described above for uninfected DRG neurones and are shown in figure 5.4. Incubation of wt HSV 17<sup>+</sup> infected DRG neurones for 24 hours with inhibitors of internalisation did not cause a shift in mean normalised sodium current-voltage relationships.
Figure 5.4. The effect of wt HSV 17+ infection of DRG neurones in the presence of internalisation inhibitors on the current-voltage relationship. The graph shows the normalised current amplitudes ± s.e.m from control (●) n=5, and from wt HSV 17+ infected DRG neurones whilst being treated with, bafilomycin A1 (▲) n=9, chloroquine (▼) n=3, and ammonium chloride (■) n=5.

For each family of sodium currents generated the conductance was calculated and the normalised conductances (g/gmax) were plotted against command potential, (figure 5.5). The activation curves of the wt HSV 17+ infected DRG neurones treated with internalisation inhibitors overlay each other and there is no significant difference in the mean slope factor and V_{50} values. This data is displayed in table 5.1, page 175.
The loss of sodium conductance which is usually associated with a wt HSV 17+ infection of DRG neurones was not observed when the infected cells were incubated in the presence of chemicals which inhibit the internalisation of membrane proteins. The presence of internalisation inhibitors did not alter the normalised sodium current amplitude or the voltage dependence of the sodium currents evoked from infected or uninfected DRG neurones.

The pH of the media containing the internalisation inhibitors was measured. The media containing chloroquine changed in pH from pH 7.4 to pH 7.0 (a 3 fold increase in H⁺ concentration). The other internalisation inhibitors ammonium chloride and bafilomycin A₁, did not alter the pH of the media.
Inhibitors of viral envelopment

Chloroquine and ammonium chloride are known to inhibit the process of maturation but not the replication of HSV-1 (Koyama & Uchida, 1984). It was, therefore, important to determine whether loss of sodium conductance was due to the inhibition of the maturation or the inhibition of internalisation. Brefeldin A inhibits viral maturation (envelopment) without affecting the internalisation pathways of cells (Koyama & Uchida, 1994). Treatment with brefeldin A causes the reversible collapse of the trans-Golgi network. This has a profound effect on the secretory pathway, whilst the endocytotic pathway remains unperturbed. DRG neurones were infected with wt HSV 17 and incubated for 1 hour, to allow adsorption and penetration of wt HSV 17, and then incubated in the presence of 1μM brefeldin A for 24 hours. Sodium currents were generated from a holding potential of -80mV to +10mV for 13 msec. Characteristic sodium currents from this set of experiments are shown in figure 5.6a. The current amplitudes were normalised to neuron size (nA/pF) and the mean amplitudes are shown in figure 5.6b. wt HSV 17 infected DRG neurones incubated in the presence of brefeldin A lose sodium conductance as observed with untreated wt HSV 17 infected neurones. Interestingly, 70% wt HSV 17 infected DRG neurones did not have a sodium current. 71% of wt HSV 17 infected DRG neurones incubated with brefeldin A also did not have a sodium current. Thus the presence of brefeldin A does not alter the ability of the virus to reduce sodium current amplitudes. This result suggests that chloroquine and ammonium chloride prevent loss of sodium conductance via inhibition of the internalisation pathway rather than via inhibition of viral maturation.
Figure 5.6. The effect of trans-Golgi network collapse on normalised sodium current amplitude of control and wt HSV 17+ infected DRG neurones. a Sodium currents of DRG neurones either treated with brefeldin A and/or infected with wt HSV 17+. b Mean normalised sodium current amplitude ± s.e.m for infected and uninfected DRG neurones with or without brefeldin A. Treatment with brefeldin A did not significantly alter the size of the sodium current amplitudes of infected or uninfected neurones. The normalised amplitudes of Brefeldin A treated HSV infected and HSV infected DRG neurones were both significantly smaller than the amplitudes of brefeldin A treated or control DRG neurones, using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.001. 70% of wt HSV 17+ infected DRG neurones had no sodium current, whilst 71% of cells infected with wt HSV 17+ and treated with brefeldin A had no sodium current.
It was necessary to ensure that the decrease of sodium current amplitudes observed from wt HSV 17+ infected DRG neurones treated with brefeldin A was a result of decreased sodium conductance rather than a shift in the current-voltage relationship. A family of sodium currents were therefore generated, as described previously. The mean normalised current-voltage relationships for DRG neurones treated with brefeldin A or wt HSV 17+ infected for 24 hours in the presence brefeldin A are shown in figure 5.7. There was no apparent shift in the current-voltage relationships which could account for the decreased current amplitudes observed at +10mV from DRG neurones infected with wt HSV 17+ in the presence of brefeldin A.

**Figure 5.7.** The effect of disruption of the trans-Golgi on the current-voltage relationship of DRG neurones. The graph shows the normalised current amplitudes ± s.e.m from DRG neurones during treatment with brefeldin A (◊) n=3, or treatment with brefeldin A (◆) n= 5, during infection with wt HSV 17+.

Individual activation curves were constructed for each brefeldin A treated and wt HSV 17+ infected DRG neurone that showed a sodium current remaining. The conductance was normalised with respect to the maximum conductance (g/gmax) and plotted against command potential. The conductance-voltage relationships are shown in figure 5.8. Comparison of the activation curves of brefeldin A treated and wt HSV 17+
infected in the presence of brefeldin A suggests no difference in the voltage
dependence of the sodium currents. This is confirmed by analysis of the mean slope
factor and $V_{50}$ values displayed in table 5.1, page 175.

**Figure 5.8.** The effect of disruption of the *trans*-Golgi on the conductance-voltage relationship of DRG neurones. The graph shows the normalised conductance ± s.e.m from DRG neurones during treatment with brefeldin A (◇) n=3, or treatment with brefeldin A (◆) n=5, during infection with wt HSV 17+. The continuous lines were obtained by fitting the mean normalised data to a Botlzmann function.

As brefeldin A and bafilomycin A₁ are made up in DMSO, solvent control experiments were carried out to determine if the presence of DMSO in the media inhibited the viral effect on sodium currents.

Infected and uninfected DRG neurones were treated with DMSO for 24 hours. Sodium currents were evoked by the one step depolarisation protocol and the currents generated were normalised for cell size (nA/pF). The mean data is shown in figure 5.9. Uninfected DRG neurones incubated in the presence or absence of DMSO had comparable current amplitudes. DMSO treated neurones infected with wt HSV 17+
showed the same reduction in sodium current amplitudes as the wt HSV 17\(^+\) infected DRG neurones. All the DRG neurones treated with DMSO had a sodium current, whilst only 1 out of 10 wt HSV 17\(^+\) infected DRG neurones treated with DMSO had a sodium current. This finding indicates that DMSO does not inactivate the virus.

**Figure 5.9. The effect of solvent (DMSO) on normalised sodium current amplitude of control and wt HSV 17\(^+\) infected DRG neurones.** The graph shows the mean normalised current amplitude ± s.e.m of control or wt HSV 17\(^+\) infected treated with DMSO solvent (1µl in 200µl of medium) DRG neurones. There is no significant difference between the current amplitudes of control in the presence or absence of DMSO or wt HSV 17\(^+\) infected DRG neurones in the presence or absence of DMSO. The current amplitudes of wt HSV 17\(^+\) infected DRG neurones in the presence or absence of DMSO were significantly smaller than the current amplitudes of uninfected cells in the presence or absence of DMSO (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.0001).

There were insufficient wt HSV 17\(^+\) infected DRG neurones either in the presence or absence of DMSO that had a sodium current remaining and thus families of sodium currents could not be generated, however all the uninfected cells had sodium currents. Figure 5.10 shows the normalised current-voltage relationship of control and DMSO...
treated neurones. The curves for control and DMSO treated neurones overlap indicating that there was no shift in the current-voltage relationships.

Figure 5.10. Current-voltage relationship for sodium currents of control and DMSO treated DRG neurones. The graph shows the mean normalised current amplitudes ± s.e.m from control (●) n= 5, and DMSO treated (○) n= 8, DRG neurones.

This result is supported by analysis of the conductance-voltage relationships for DMSO treated uninfected DRG neurones shown in figure 5.11. Comparison of the curves and the calculated $V_{50}$ and slope factors for fitted Boltzmann relationships, shown in table 5.1, show that the presence of DMSO does not affect the voltage dependence of sodium currents.
Figure 5.11. Conductance-voltage relationship for sodium channels of control and DMSO treated DRG neurones. The graph shows the mean normalised conductance ± s.e.m from control (●) n= 5, and DMSO treated (○) n= 8, DRG neurones. The continuous lines were obtained by fitting the mean normalised data to a Boltzmann function.
Table 5.1. Electrophysiological characteristics of control DRG neurones and wt HSV 17^+ infected treated with inhibitors of internalisation. The slope factors and the voltage at half maximal conductance ($V_{50}$) were not significantly different between the groups (Tukey’s pairwise post hoc comparison following analysis of variance $P>0.4$). $V_{50}$ and slope factors ± s.e.m. were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each neurone.

In summary, the loss of sodium conductance observed after 24 hours of infection of DRG neurones with wt HSV 17^+ can be prevented by inhibiting membrane internalisation pathways. These results can not be explained by the presence of the solvent DMSO or by the fact that some of the internalisation inhibitors used also prevented the viral maturation process of envelopment.
Western blot analysis

The possibility that sodium channels were lost from DRG neurones after wt HSV 17+ infection was investigated by Western blot analysis. The DRG neurone cultures were treated with cytosine arabinoside for 2 day intervals, for two weeks, to eliminate rapidly dividing non-neuronal cells. After two weeks, when the cultures were purely neuronal, the cytosine arabinoside was removed and neurones were infected with wt HSV 17+. The same quantity of cell protein from infected, uninfected DRG neurones and Chinese hamster ovary (CHO) cells were added to each lane of the SDS-polyacrylamide gel. The proteins were separated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose filter. The CHO cell protein is known not to contain voltage-gated sodium channels and was used as a control. The blot was probed with an antibody directed against the highly conserved region of the intracellular III-IV loop of sodium channel α subunits (Upstate Biotechnology Incorporated). The first two lanes of the panel of figure 5.12 contained proteins from infected and uninfected DRG neurones: protein bands of 220kDa, the expected size of α subunit, were visible. The third lane of the panel contained cell proteins of CHO cells, no band corresponding to sodium channel α subunit was observed as expected. Comparison of the protein bands corresponding to sodium channel α subunit of wt HSV 17+ infected and uninfected DRG neurones does not show any obvious difference in protein levels.
Figure 5.12. Western blot analysis of the sodium channel α subunits from wt HSV 17+ infected DRG neurones, uninfected DRG neurones and CHO cells. Cell proteins were separated by SDS-PAGE and analysed by Western blotting. The filters were probed with anti-sodium channels antibody. Proteins recognising the antibody were visualised by enhanced chemiluminescence. The panel shows protein bands at 220kDa for the wt HSV 17+ infected and uninfected DRG neurones. This Western blot is representative of three experiments.

Immunohistochemistry

The distribution of sodium channels of wt HSV 17+ infected and uninfected DRG neurone populations were investigated by immunofluorescence. Permeabilised DRG neurones were incubated with an antibody directed against the highly conserved region of the intracellular III-IV loop of the sodium channel α subunit. The sodium channels were visualised by indirect immunofluorescence (see methods). Figure 5.13a shows the pattern of staining of sodium channel α subunits in a control DRG neurone and figure 5.13b shows the immunofluorescent staining of wt HSV 17+ infected DRG neurones. Control neurones showed a diffuse staining pattern while punctate staining was observed in most of the wt HSV 17+ infected DRG neurones. This could reflect internalisation of sodium channels into the endosomes.
Figure 5.13 The distribution of sodium channels of HSV infected and uninfected DRG neurones. a An uninfected DRG neurone x 600 showing a diffuse staining pattern. b The punctate distribution of sodium channels in HSV infected DRG neurones. The distribution of sodium channels was visualised by indirect fluorescin-fluorescence under blue illumination.

The distribution of potassium and calcium channels of wt HSV 17 infected DRG neurones was investigated for comparison with the altered sodium channels distribution. The distribution of potassium and calcium channels did not appear punctate after 24 hours infection with wt HSV 17 (figure 5.14a and b).
Figure 5.14. The distribution of potassium and calcium channels in HSV infected DRG neurones. a The diffuse staining pattern of potassium channels in DRG neurones after 24 hours of wt HSV 17\textsuperscript{*} infection visualised by indirect immunofluorescence. b The diffuse staining pattern of calcium channels in DRG neurones after 24 hours of wt HSV 17\textsuperscript{*} infection visualised by indirect immunofluorescence.
The possibility that sodium channel $\alpha$ subunits were located within acid endosomes of wt HSV 17$^+$ infected DRG neurones was investigated using a red dye called Lysotracker (Molecular Probes). The Lysotracker dye that stains acid endosomes red was used to compare the staining pattern acid endosomes of infected and uninfected DRG neurones. Figure 5.15a shows the staining of acid endosomes of wt HSV 17$^+$ infected and uninfected DRG neurones. The acid endosomes staining pattern compares well with that of the sodium channel $\alpha$ subunits of wt HSV 17$^+$ infected DRG neurones (figure 5.15b). These observations suggest that the sodium channel $\alpha$ subunit of wt HSV 17$^+$ infected DRG neurones may be located in acid endosomes, which would be consistent with the hypothesis that sodium channels were internalised following a lytic infection with wt HSV 17$^+$.

Figure 5.15. Comparison of the staining pattern of sodium channels in HSV infected DRG neurones with the location of neuronal acid endosomes. a The pattern of acid endosomes in uninfected DRG neurones. b The staining pattern of sodium channels from HSV infected DRG neurone.
Prolonged sodium channel opening

The loss of sodium conductance associated with wt HSV 17\(^+\) infection of DRG neurones was prevented by blocking plasma membrane internalisation pathways. This indicates that sodium channels are lost from the plasma membrane via internalisation. It is interesting to consider what mechanism or trigger causes the internalisation process to occur after 24 hours of viral infection. Previous studies of neonatal rat brain, but not adult rat brain, have shown that internalisation of sodium channels very rapidly follows a rise in intracellular sodium concentration evoked by an influx of ions through voltage-gated sodium channels or via a sodium ionophore such as amphotericin B (Dargent et al. 1994). The following series of experiments were designed to investigate whether sodium channels of wt HSV 17\(^+\) infected DRG neurones were internalised following a rise in intracellular sodium concentration.

The sodium channel activator veratridine shows state or use-dependent binding, as the binding site is located within the pore and only becomes accessible when the channel is open. Binding of veratridine to open sodium channels results in prolonged channel opening. Unlike normal opening of sodium channels which lasts for milliseconds, the veratridine modified openings last for seconds. Voltage-clamp experiments, in the whole-cell mode, showed that the sodium currents activate normally during depolarisation, but failed to inactivate at the end of the depolarisation step, instead persisting for a couple of seconds after return to the resting membrane potential (Hille, 1968).

Depolarising neuronal membranes with 50mM KCl increases sodium channel open probability and thus the probability of veratridine binding is also increased. Incubation of DRG neurones with 50mM KCl and 0.1mM veratridine, results in the prolonged opening of sodium channels.

One explanation for the loss of sodium conductance during a wt HSV 17\(^+\) infection could be that sodium channels are internalised following a rise in the intracellular sodium concentration. The effects of increased intracellular sodium concentration in DRG neurones were therefore investigated.
DRG neurone cultures were incubated in the presence of 50mM KCl and 0.1 mM veratridine for 5 or 24 hours. The veratridine was then completely washed out by rinsing the neurones 3 times, each for 7 minutes in fresh prewarmed F-14 medium before transferring the coverslip to the recording chamber in fresh extracellular recording solution. The sodium currents were evoked by the one step depolarisation protocol (figure 5.16a). The mean normalised currents are displayed in figure 5.16b.
Figure 5.16. The effect of prolonged sodium channel opening on normalised sodium current amplitude of dorsal root ganglion neurones. 

a Sodium current traces from control neurones and neurones treated with veratridine and 50mM KCl for 5 or 24 hours. The leak currents were subtracted. 

b The normalised sodium current ± s.e.m is shown for control neurones and neurones treated with both 0.1 mM veratridine and 50mM KCl for 5 hours and 24 hours. The normalised sodium current amplitudes from veratridine and KCl treated neurones at 5 and 24 hours were both significantly smaller than the control, **P<0.05, but not significantly different from each other, P>0.01. Statistical differences were examined with the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison.

The normalised sodium current amplitudes of 50mM KCl and 0.1 mM veratridine for 5 or 24 hours were both significantly smaller than untreated, control neurone currents. 14% of cells tested after 5 hours showed no sodium current whereas 70% of neurones tested after 24 hours had no sodium current. Despite the difference in percentage of neurones with a current, the difference between the normalised currents recorded after 5 or 24 hours incubation was not significant. As a result, in subsequent experiments, data from cells incubated between 5 and 24 hours were grouped together.
The results indicate that a treatment that prolongs sodium channels opening, and consequently increases the intracellular sodium concentration, results in a loss of sodium conductance in DRG neurones.

Figure 5.17 shows the normalised current-voltage relationship for control and 50mM KCl and veratridine treated neurones that showed a sodium current.

**Figure 5.17. The effects of prolonged sodium channel opening on the sodium current-voltage relationship of DRG neurones.** The graph shows the normalised sodium current ± s.e.m plotted against the command potential for control (●) n=9, and 0.1mM veratridine and 50mM KCl incubation for 5 hours (■) n=12, or 24 hours (□) n=9. Currents at each potential were evoked following a hyperpolarising prepulse to -120mV.

There was no apparent shift in the normalised current-voltage relationships from cells incubated with 50mM KCl and 0.1 mM veratridine for 5 or 24 hours compared with the control. Conductance-voltage relationships were constructed for each DRG neurone and the averaged data are plotted in figure 5.18.
Figure 5.18. The effect on the sodium conductance-voltage relationship of DRG neurones after prolonged sodium channel opening. The graph shows the normalised conductance ± s.e.m plotted against command potential for control (●) n=9, and 0.1 mM veratridine and 50 mM KCl incubation for 5 hours (■) n=12, or 24 hours (□) n=9.

The averaged $V_{50}$ and slope factor values taken from individual activation curves are shown in table 5.2. These results illustrate that the sodium channel activation properties were not modified in 50 mM KCl and veratridine treated cells if they retained a sodium current.
Table 5.2. Electrophysiological characteristics of sodium conductances from DRG neurones treated with sodium channel opening agents. The slope factors and the voltage at half maximal conductance (V₅₀) were not significantly different between the groups, P>0.09 (analysis of variance followed by Tukey's post hoc analysis). V₅₀ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each neurone.

There was no significant difference between the electrophysiological characteristics of the sodium currents remaining in neurones that had been treated with sodium channel activators for 5 or 24 hours and those in control DRG neurones.

Immunohistochemistry

The distribution of sodium channels of veratridine and KCl treated DRG neurones were investigated by immunofluorescence. Permeablised DRG neurones were incubated with the sodium channel antibody described previously (see methods). Figure 5.19a shows the staining pattern of sodium channel α subunits of control DRG neurones, whilst figure 5.19b shows the pattern of channels of DRG neurones incubated with veratridine and KCl for 5 hours. The punctate staining of the veratridine and KCl treated DRG neurones could be sodium channels along the internalisation pathway. This punctate pattern of sodium channel staining was similar to that observed in DRG neurones after 24 hours of wt HSV 17^ infection.
Figure 5.19. The effect that prolonged sodium channel activation has on their distribution in DRG neurones. a The pattern of sodium channel staining of a DRG neurone. b The localisation of sodium channels following treatment with veratridine and KCl that prolonged channel opening. The neurones were visualised by indirect immunofluorescence under u.v light, images are x 400.

In summary, DRG neurones incubated in 50mM KCl and 0.1mM veratridine for 5-24 hours had significantly reduced sodium currents compared to control currents. A prepulse to -120 mV did not reveal a sodium current in neurones which had not shown a current after the one step depolarisation protocol. The reduced sodium current following prolonged sodium channel opening suggests that an increased intracellular sodium concentration could cause a loss of sodium channels from DRG neurone plasma membranes. The punctate staining of sodium channel α subunits after 5 hours veratridine plus KCl treatment also suggests that sodium channels were internalised following prolonged sodium channel opening.
Block of sodium ion influx

It has been demonstrated that sodium channel down regulation from neonatal rat neurones occurs after treatment with sodium channel activators such as veratridine (Dargent & Couraud, 1990). The loss of sodium channels from the surface of neonatal rat neurones was triggered by the influx of sodium ions. If, however, the influx of sodium ions was blocked, either by conducting the experiments in sodium free media or in the presence of the sodium channel blocker TTX, the loss of surface sodium channels was prevented (Paillart et al. 1996).

The previous section entitled “prolonged sodium channel opening”, has shown that sodium conductance is lost from adult rat DRG neurones after treatment with veratridine and it is possible that this loss of sodium conductance is also due to sodium ion influx followed by channel down regulation. The experiments described in this section investigate the possibility that sodium entry into DRG neurones during treatment with veratridine results in the loss of sodium conductance.

TTX inhibits TTX-S sodium channels at nanomolar concentrations, leaving the TTX-R sodium channels fully functional. At mM concentrations, however, both TTX-S and TTX-R sodium channels are blocked (see table 1.2). In these experiments 5 mM TTX was used, which was sufficient to block both TTX-S and TTX-R sodium channels. DRG neurone cultures were incubated with of 0.1 mM veratridine and 50 mM KCl in the presence or absence of the sodium channel blocker 5 mM TTX. After 24 hours of incubation the cells were washed thoroughly (3 washes in fresh medium for 7 minutes) and sodium currents were elicited by the one step depolarisation protocol (figure 5.20a). The mean normalised current amplitudes for veratridine and 50 mM KCl treated neurones were reduced compared to DRG neurones treated with veratridine, 50 mM KCl and TTX (figure 5.20b). The addition of TTX was sufficient to prevent the loss of sodium conductance observed when DRG neurones were incubated in the presence of sodium channel activators. This suggests that sodium influx through voltage-gated sodium channels triggers loss of sodium conductance observed after treatment with veratridine in depolarising media. All DRG neurones treated with
TTX, veratridine and KCl had a sodium current, whereas 67% DRG neurones in the presence of only veratridine and KCl had no sodium current. This indicated that the presence of TTX is sufficient to prevent the loss of sodium currents usually associated with DRG neurones treated with veratridine and KCl.
Figure 5.20. The effect of prolonged sodium channels opening in the presence or absence of a sodium channel blocker TTX. a Leak subtracted sodium current traces evoked by a depolarisation from -80mV to +10mV of veratridine treated DRG neurones with or without TTX present during incubation. b The normalised sodium current ± s.e.m is shown for control DRG neurones and DRG neurones treated with both 0.1 mM veratridine and 50mM KCl in the presence or absence of TTX for 5-24 hours. The normalised sodium current amplitudes of control or veratridine and KCl treated in the presence of TTX DRG neurones were not significantly different from each other (P>0.2) but were both significantly different to cells treated with veratridine and KCl (P<0.001) (Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison).

Figure 5.21 shows the normalised current-voltage relationships of control, veratridine and 50mM KCl incubated in the presence or absence of TTX and figure 5.22 shows the corresponding normalised conductance-voltage relationships.
Figure 5.21. The effects of blocking sodium channels during incubation with veratridine on the sodium current-voltage relationship of DRG neurones. The graph shows the normalised sodium current ± s.e.m plotted against command potential for control (●) n=8, 0.1mM veratridine and 50mM KCl for 24 hours (□) n=20 and 0.1mM veratridine and 50 mM KCl with TTX present (■) n=18. Currents were evoked from a prepulse potential of -120mV.

The mean $V_{50}$ and slope factor values calculated from each individual activation curve are displayed in table 5.3. There was no significant difference in the voltage dependence of the sodium currents recorded in these conditions.
Figure 5.22. The effect on the conductance-voltage relationship of blocking sodium channels with TTX whilst incubating DRG neurones with veratridine and KCl. The graph shows the conductance-voltage relationship ± s.e.m for control (●) n=8, 0.1mM veratridine and 50mM KCl for 24 hours (□) n=20 and 0.1mM veratridine and 50 mM KCl with TTX present (■) n=18. The curves were obtained by fitting the data points from each neurone with a Boltzmann function.

<table>
<thead>
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<th>V_50 (mV)</th>
<th>slope factor (mV)</th>
<th>number of cells</th>
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<tbody>
<tr>
<td>control</td>
<td>-19.8 ± 1.4</td>
<td>4.7 ± 0.5</td>
<td>8</td>
</tr>
<tr>
<td>veratridine+50mM KCl 5-24 hours</td>
<td>-21.3 ± 2.3</td>
<td>5.8 ± 0.7</td>
<td>20</td>
</tr>
<tr>
<td>veratridine+50mM KCL+TTX 24 hours</td>
<td>-22.2 ± 2.5</td>
<td>5.6 ± 0.8</td>
<td>18</td>
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Table 5.3. Electrophysiological characteristics of sodium conductances from DRG neurones treated with channel opening and blocking agents. The slope factors and the voltage at half maximal conductance (V_50) were not significantly different between the groups (analysis of variance followed by Tukey’s post hoc analysis). V_50 and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each neurone.
The blockade of sodium influx inhibits the loss of sodium conductance in DRG neurones evoked by treatment with veratridine and 50mM KCl. This finding suggests that the loss of sodium conductance can be dependent on sodium ion influx and raises the possibility that the loss of sodium conductance from wt HSV 17\(^+\) infected DRG neurones observed after 24 hours was also triggered by sodium influx. This was investigated by preventing sodium influx into DRG neurones infected with wt HSV 17\(^+\).

Infected and uninfected DRG neurone cultures were incubated in the presence or absence of TTX. After 24 hours the cultures were washed thoroughly, 3 times, with fresh medium to remove TTX before electrophysiological study. Sodium currents were generated by a step depolarisation to +10mV. Examples of typical recordings are shown in figure 5.23a. The current amplitudes were normalised for cell size (nA/pF) and are displayed in figure 5.23b. After 24 hours of infection with wt HSV 17\(^+\), DRG neurones had a dramatically reduced sodium current. This loss, however, did not occur when TTX had been present in the media. There was no significant difference in the normalised sodium current amplitudes of infected or uninfected DRG neurones, incubated in the presence or absence of TTX, for 24 hours. All control and TTX treated DRG neurones had a sodium current. 75% of wt HSV 17\(^+\) infected, TTX treated DRG neurones had a sodium current. In contrast, only 14% of wt HSV 17\(^+\) infected DRG neurones had a sodium current. This shows that although the presence of TTX does prevent the loss of sodium current amplitude in wt HSV 17\(^+\) infected DRG neurones, there were still some DRG neurones (2/8) with no sodium current.
Figure 5.23. The effect of sodium channel block during a wt HSV 17+ infection on sodium current amplitude of DRG neurones. a Sodium current traces evoked by a one step depolarisation to +10mV. The leak subtracted currents were from infected or uninfected DRG neurones treated with TTX for 24 hours. b The graph shows the normalised current amplitude ± s.e.m of control, TTX treated, wt HSV 17+ infected, wt HSV 17+ infected and TTX treated DRG neurones. The normalised current amplitude of wt HSV 17+ infected cells was found to be significantly different from the other conditions tested, P<0.0001 (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison).

The normalised sodium current-voltage relationships from control, TTX treated and wt HSV 17+ infected in the presence of TTX, DRG neurones are shown in figure 5.24.
Figure 5.24. The effect of blocking sodium channels with TTX during a wt HSV 17+ infection of DRG neurones. The graph shows the normalised current-voltage relationship ± s.e.m of control (●) n=8, TTX treated (□) n=5 and wt HSV 17+ infected during treatment with TTX (■) n=5.

The normalised conductance-voltage relationships for each neurone tested are shown in figure 5.25. The mean fractional conductance (g/gmax) plotted against command potential for control, TTX treated and wt HSV 17+ infected in the presence of TTX, DRG neurones is shown in figure 5.25. The activation curve of wt HSV 17+ infected DRG neurones incubated in TTX are shifted to more positive potentials compared to the other curves. This shift may be indicative of predominantly TTX-R currents which activate at more depolarised potentials than TTX-S currents. Despite this apparent shift in activation the mean \( V_{50} \) and slope factor values shown in table 5.4, do not show a statistically significant difference.
Figure 5.25. The conductance-voltage relationship from control and wt HSV 17\(^+\) infected DRG neurones treated with the sodium channel blocker TTX. The normalised conductance ± s.e.m for control (●) n=8, TTX treated (□) n=5 and wt HSV 17\(^+\) infected during treatment with TTX (■) n=5 plotted against command potential, is shown. The curves were obtained by fitting the data points from each neurone with a Boltzmann function.
Table 5.4. The slope factor and half maximal values ($V_{50}$) of sodium conductances from control, TTX pretreated and TTX treated during wt HSV 17$^+$ infection of DRG neurones. The slope factors and $V_{50}$ values were not significantly different between the groups, $P>0.09$ (analysis of variance followed by Tukey's post hoc analysis). $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each neurone.

The blockade of the voltage-gated sodium channels of DRG neurones with TTX was sufficient to prevent the loss of sodium conductance, observed in wt HSV 17$^+$ infected DRG neurones after 24 hours. A possible reason for this effect, based on the work of Dargent et al (1990), was that TTX blocked sodium influx, so that the intracellular sodium concentration did not rise during wt HSV 17$^+$ infection and sodium channel internalisation was not triggered.

This possibility was investigated by preventing sodium influx into DRG neurones by removing sodium from the extracellular medium. For these experiments, DRG neurones were incubated in a modified tissue culture medium containing choline instead of sodium. After 24 hours of incubation infected or uninfected cultures were placed in solution appropriate for recording isolated sodium currents (chapter 2, solutions and voltage protocols, page 47) and the sodium currents were evoked by the one step depolarisation protocol. Representative sodium currents are illustrated in figure 5.26a. The normalised sodium currents from infected and uninfected cells incubated in a sodium free media are displayed in figure 5.26b. They were compared to currents from sister cultures incubated in a normal tissue culture medium.

<table>
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<tr>
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<th>$V_{50}$ (mV)</th>
<th>slope factor (mV)</th>
<th>number of cells</th>
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<tr>
<td>control</td>
<td>-18.8 ± 2.4</td>
<td>3.2 ± 0.5</td>
<td>8</td>
</tr>
<tr>
<td>TTX 24 hours</td>
<td>-19.5 ± 3.2</td>
<td>3.3 ± 0.5</td>
<td>7</td>
</tr>
<tr>
<td>wt HSV 17$^+$ and TTX 24 hours</td>
<td>-14.1 ± 1.8</td>
<td>4.7 ± 0.5</td>
<td>5</td>
</tr>
</tbody>
</table>
No significant difference was noted between the sodium currents in infected and uninfected DRG neurones cultured in sodium free media, (P>0.7). This is in contrast to the findings for infected and uninfected DRG neurones cultured in normal F-14 media, where the normalised sodium current amplitudes of wt HSV 17<sup>+</sup> infected DRG neurones were significantly smaller than those of control cells, (P<0.0002). The currents of the infected and uninfected DRG neurones cultured in sodium free media were significantly larger than the normalised amplitudes of wt HSV 17<sup>+</sup> infected DRG neurones incubated in control conditions, (P<0.05). The prevention of sodium influx into DRG neurones during a wt HSV 17<sup>+</sup> infection inhibited the loss of sodium conductance associated with viral infection. The results were not simple to interpret, however, as the sodium currents of control DRG neurones (incubated in normal F-14) were significantly larger than the normalised sodium current amplitudes of DRG neurones incubated in sodium free media, (P<0.002). The reason for this decrease is unknown. After incubation of DRG neurones in sodium free media, 23% of neurones had no measurable sodium current. A similar percentage (25%) of wt HSV 17<sup>+</sup> infected DRG neurones incubated in sodium free media, had no sodium current. In contrast, the proportion of wt HSV 17<sup>+</sup> infected DRG neurones incubated in media containing sodium, without a sodium current, was 87%. It is interesting to note that the percentage of infected DRG neurones that showed no sodium current after incubation in sodium free media, was similar to the proportion of uninfected neurones also incubated in sodium free media showing no sodium current. Whereas, a larger percentage of infected DRG neurones incubated in sodium containing media showed no sodium current compared to uninfected DRG neurones also incubated in sodium containing media.
Figure 5.26. The effect on sodium current amplitudes of incubating DRG neurones in sodium free media during wt HSV 17⁺ infection. a Sodium currents from infected and uninfected DRG neurones incubated in sodium free media. The leak currents were subtracted. b The graph shows the normalised sodium current ± s.e.m of control and wt HSV 17⁺ infected neurones in the presence or absence of sodium free media. The sodium currents from the control neurones were significantly larger than the amplitudes from the other neurones tested (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.001). However considering only the neurones treated in sodium free media, the current amplitudes from wt HSV 17⁺ infected cells were significantly different than those from uninfected neurones (using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison, P<0.05).

Normalised current-voltage relationship (figure 5.27) and conduction-voltage curves (figure 5.28) were constructed for each family of sodium currents generated. Comparison of the activation curves and the $V_{50}$ and slope factor values (table 5.5) show that there are no significant changes in the voltage dependence of the sodium currents evoked from DRG neurones incubated for 24 hours in the absence of sodium.
Figure 5.27. The effects on the sodium current-voltage relationship of incubating DRG neurones in sodium free medium during a wt HSV 17+ infection. The graph shows the normalised sodium current ± s.e.m plotted against command potential for neurones incubated in sodium free media (■) n=11, for neurones incubated in sodium free media during a wt HSV 17+ infection (□) n=19 and control neurones (●) n=8.
Figure 5.28. The effect on the sodium conductance-voltage relationship from DRG neurones incubated in sodium free media in control or wt HSV 17+ infected conditions. The normalised conductance ± s.e.m of neurones incubated in sodium free media (■) n=11, for neurones incubated in sodium free media during a wt HSV 17+ infection (□) n=19 and control neurones (●) n=8 is plotted against command potential. The curves were obtained by fitting the data points from each DRG neurone with a Boltzmann function.

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</tr>
<tr>
<td>sodium free</td>
<td>-17.8 ± 1.6</td>
<td>7.5 ± 1.6</td>
<td>11</td>
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<tr>
<td>sodium free + wt HSV 17+</td>
<td>-19.7 ± 3.5</td>
<td>6.4 ± 0.5</td>
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Table 5.5. Electrophysiological characteristics of sodium conductances of DRG neurones incubated in sodium free media in control or wt HSV 17+ infected conditions for 24 hours. The slope factors and the voltage at half maximal conductance ($V_{50}$) were not significantly different between the groups (analysis of variance followed by Tukey's post hoc analysis). $V_{50}$ and slope factor values were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each DRG neurone.
These results demonstrated that preventing sodium influx into wt HSV 17+ infected DRG neurones by incubating DRG neurones in sodium free media inhibited the loss of sodium conductance associated with wt HSV 17+ infection of DRG neurones. This finding suggests that sodium influx was important in the process of sodium conduction loss and was perhaps involved in the triggering of internalisation of sodium channels.

**Controls**

**Effects of either veratridine or KCl alone**

In order to illustrate that treating DRG neurones with 50mM KCl and 0.1 mM veratridine causes loss of sodium conductance, the following sets of control experiments were performed. DRG neurones were incubated with either 0.1mM veratridine or 50mM KCl for 24 hours. This was then completely washed out with 3 separate 7 minute washes. In other experiments, 0.1mM veratridine was applied acutely to the bath immediately prior to recording. This was designed to investigate the effect of direct application of veratridine on sodium currents generated. The sodium currents were then evoked with the one step depolarisation protocol and were normalised for neurone size. There was no significant difference in the normalised sodium current amplitudes from neurones incubated with 50mM KCl or 0.1mM veratridine, or from neurones treated acutely with 0.1mM veratridine (figure 5.29). All DRG neurones tested had a sodium current.
Figure 5.29. The effects on sodium current amplitude of incubating DRG neurones with 0.1mM veratridine or 50 mM KCl. The graph shows normalised sodium current ± s.e.m for 0.1mM veratridine treated DRG neurones for either 24 hours or acute treatment, 50 mM KCl treated DRG neurones and control DRG neurones. There was no significant difference between the normalised sodium current amplitudes of DRG neurones under these experimental conditions (ANOVA followed by Tukey’s post hoc analysis, P>0.3).

Incubating DRG neurone cultures for 24 hours with the sodium channel activator veratridine alone or with 50mM KCl alone, was not sufficient to induce loss of sodium conductance. Exposure to a combination of both, however, evoked a loss of sodium current. These findings suggested that 50mM KCl is required to depolarise DRG neurone membranes. This allows veratridine to gain access to its binding site within open sodium channels. The consequent prolongation of sodium channel opening by veratridine is required for the subsequent loss of sodium conductance.

Figure 5.30 shows the normalised current-voltage curves for DRG neurones, treated as described above. The corresponding normalised conductance-voltage relationships are illustrated in figure 5.31. From the individual activation curves the $V_{50}$ and slope factor values were calculated, the mean data is shown in table 5.6.
Figure 5.30. The sodium current-voltage relationship for DRG neurones treated with 0.1mM veratridine for 24 hours or with 0.1mM veratridine acutely applied and 50 mM KCl for 24 hours. The graph shows the normalised current ± s.e.m for control (●) n=9, 0.1mM veratridine treated for 24 hours (◇) n=13, 0.1mM veratridine acutely applied (■) n=11, and 50 mM KCl treated for 24 hours (○) n=12 DRG neurones.

The activation curves for sodium currents from DRG neurones acutely treated with 0.1mM veratridine was shifted in the hyperpolarising direction compared to the control curve. There was also a leak current resulting from spontaneously opening sodium channels staying open for longer in the presence of veratridine. In contrast, no obvious shift in the activation curve was noted with the other treatments.
Figure 5.31. The conductance-voltage relationship for sodium channels of DRG neurones treated with 0.1mM veratridine for 24 hours or with 0.1mM veratridine acutely applied and 50 mM KCl for 24 hours. The graph shows the normalised conductance ± s.e.m for control (●) n=9, 0.1mM veratridine treated for 24 hours (□) n=13, 0.1mM veratridine acutely applied (■) n=11, and 50 mM KCl treated for 24 hours (〇) n=12 DRG neurones. The curves were obtained by fitting the data points from each DRG neurone with a Boltzmann function.

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<td>9</td>
</tr>
<tr>
<td>0.1mM veratridine 24 hours</td>
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<td>7.0 ± 0.8</td>
<td>13</td>
</tr>
<tr>
<td>0.1mM veratridine acute</td>
<td>-30.4 ± 2.4</td>
<td>5.2 ± 0.7</td>
<td>11</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>-22.1 ± 3.3</td>
<td>6.3 ± 0.8</td>
<td>12</td>
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Table 5.6. The electrophysiological characteristics of sodium conductances generated from DRG neurones treated with veratridine or KCl. The slope factors and the voltage at half maximal conductance ($V_{50}$) were not significantly different between the groups, $P>0.3$ (analysis of variance followed by Tukey’s post hoc analysis). $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each DRG neurone.
These results show that the loss of sodium current caused by incubating cells with 50mM KCl and 0.1mM veratridine could not be explained as an effect of 50mM KCl or 0.1mM veratridine alone. Veratridine applied acutely did not cause a loss of sodium conductance or mask the appearance of voltage activated currents evoked by the voltage protocols used for routine experimentation. The loss of sodium conductance cannot therefore be explained by the inefficient washing out of veratridine.
Calcium and potassium currents

To establish whether the loss of sodium current seen following treatment with veratridine and KCl was specific for sodium channels, the effect of veratridine and KCl treatment on other ion channels of DRG neurones was investigated. DRG neurone cultures were incubated in 0.1mM veratridine and 50mM KCl for 24 hours, washed as described above and calcium and potassium currents were studied using appropriate solutions to isolate these currents (see methods).

Calcium currents were evoked by a step depolarisation from a holding potential of -80mV to a command potential of +10mV and the amplitudes of the elicited currents were normalised for neurone size (nA/pF). Typical sodium currents recorded are shown in figure 5.32a. The mean normalised current amplitudes are shown in figure 5.32b for control and veratridine treated DRG neurones. There was no significant change in the normalised calcium currents of DRG neurones after treatment with 0.1mM veratridine and 50mM KCl.

a

control

veratridine and KCl

2nA

10ms

207
Figure 5.32. The effect of prolonged sodium channel opening on the calcium channel currents of DRG neurones.  

a Calcium currents evoked by a step depolarisation from -80mV to +10mV from control or veratridine and KCl DRG neurones. The leak currents were subtracted.  

b The normalised calcium currents from DRG neurones incubated in 0.1mM veratridine for 24 hours. The graph shows the normalised current amplitude at +10mV of control and veratridine treated DRG neurones. There was no significant difference between the current amplitudes Student’s t test, P>0.7.

The activation properties of the calcium currents were also studied by generating currents from a holding potential of -80mV with command potentials at 5mV intervals between -80mV and +75mV. The peak currents generated at each command potential were normalised with respect to the maximum current generated and the normalised sodium currents plotted against command potential (figure 5.34). Comparison of the current-voltage relationships of control and 0.1mM veratridine and 50mM KCl show no apparent difference. The increased currents at -40mV represents the low voltage activated component which is slightly more pronounced in the 0.1mM veratridine and 50mM KCl treated DRG neurones.
Figure 5.33. The calcium current-voltage relationship of control and 0.1mM veratridine plus 50mM KCl treated DRG neurones. The graph shows the normalised calcium current ± s.e.m plotted against the command potential from control (●) n=7, and 0.1mM veratridine and 50mM KCl treated (■) n=11 neurones.

The conductance-voltage relationships were constructed for each individual neurone tested. The mean normalised conductance (g/gmax) was plotted against command potential for control and 0.1mM veratridine plus 50mM KCl treated DRG neurones, as shown in figure 5.34. The activation curves almost overlap showing that there was no shift in the voltage dependent properties of the calcium channel currents.
Figure 5.34. Conductance-voltage relationship for calcium conductances of control or 0.1 mM veratridine treated DRG neurones. The graph shows the mean normalised conductances of control (●) n=7, and veratridine treated (■) n=11 DRG neurones. The curves were obtained by fitting the data points with a Boltzmann function.

This is confirmed by estimates of the $V_{50}$ and slope factor values for each individual activation curve. The averaged data is displayed in table 5.7, which shows that there was no significant difference in the $V_{50}$ and slope factor values of calcium currents, either from control DRG neurones or veratridine and KCl treated DRG neurones.
Table 5.7. Electrophysiological characteristics of calcium conductances of control or veratridine treated DRG neurones. The slope factors and the voltage at half maximal conductance ($V_{50}$) were not significantly different between the groups, Student's t test, $P>0.4$. $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each DRG neurone.

Although exposure of DRG neurone cultures to 0.1mM veratridine and 50mM KCl resulted in down regulation of sodium channels, this treatment had no effect on the calcium channel currents.

The effect of prolonged incubation in 0.1mM veratridine and 50mM KCl on potassium currents was investigated by evoking currents from a holding potential of -80mV. The currents evoked by a single step to +10mV were normalised to the size of the DRG neurones (nA/pF). Typical potassium current traces are shown in figure 5.35a. The mean normalised current amplitude for control and veratridine plus KCl treated DRG neurones are shown in figure 5.35b. There was no significant difference in the normalised current amplitudes between these two groups. Prolonging sodium channel open time with veratridine and KCl did not affect the normalised potassium current amplitude.
Figure 5.35. The effect of prolonged sodium channel opening on the delayed rectifier type potassium currents of DRG neurones. 

a Delayed rectifier type potassium currents of veratridine and KCl or control DRG neurones. The leak currents were subtracted.

b The effect of incubating DRG neurones in 0.1 mM veratridine and 50 mM KCl for 24 hours on the delayed rectifier type potassium current amplitudes. The normalised mean potassium current amplitude ± s.e.m is shown for control and veratridine treated DRG neurones. There was no significant difference between the normalised amplitudes of control and veratridine treated neurones, Student’s t test, P>0.07.
Families of potassium currents were also generated from a holding potential of -80mV with command potentials between +70mV and -75mV and the peak currents were used to construct current-voltage relationships which were normalised with respect to the maximum evoked conductance. The mean normalised potassium currents generated from control or 0.1mM veratridine plus 50mM KCl treated DRG neurones are shown in figure 5.36.

![Graph showing the activation curve of delayed rectifier type potassium currents.](image)

**Figure 5.36. The effect of treating DRG neurones with 0.1 mM veratridine for 24 hours on the activation curve of the delayed rectifier type potassium currents.** The graph shows the normalised conductance ± s.e.m plotted against command potential. The activation curves of control (●) n=15, and veratridine treated (■) n=5 DRG neurones are shown. The curves were obtained by fitting the data points with a Boltzmann function.

The potassium conductance-voltage relationships almost overlay and statistical analysis of the $V_{50}$ and slope factor values of each individual curve show that there is no difference in the electrophysiological characteristics of potassium currents in the two groups (table 5.8). Thus there was no difference in the voltage dependence of the potassium currents after treatments that resulted in sodium channel down regulation.
Table 5.8. Electrophysiological characteristics of the delayed rectifier type potassium conductances of control or veratridine treated DRG neurones. The slope factors and the voltage at half maximal conductance ($V_{50}$) were not significantly different between the groups, Student's $t$ test, $P>0.5$. $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each DRG neurone.

These data show that treating DRG neurones with 0.1mM veratridine and 50mM KCl results in a loss of sodium current, perhaps by internalisation of sodium channels, whilst the potassium and calcium currents remain unchanged.

Discussion

Sodium currents were lost from DRG neurones infected with wt HSV 17$^+$ after 24 hours (chapter 3). The loss in sodium conductance is the result of an event downstream of viral DNA replication (chapter 4). The mechanism for sodium current loss and particularly the possibility that sodium channel density is decreased by internalisation during a wt HSV 17$^+$ infection, was investigated in this chapter.

Incubation of wt HSV 17$^+$ infected DRG neurones in the presence of internalisation inhibitors chloroquine and ammonium chloride was sufficient to prevent the loss of sodium conductance associated with a wt HSV 17$^+$ infection. Although there was a shift in the current-voltage relationship for 2 of the 5 uninfected DRG neurones treated with 100µM chloroquine, no such change was noted for 100µM chloroquine treated wt HSV 17$^+$ infected DRG neurones. The ability of chloroquine to inhibit sodium current loss after wt HSV 17$^+$ infection cannot, therefore, be due to any shift in the activation...
properties. The shift in the current-voltage relationship is unlikely to be due to the slight acidification of media in the presence of chloroquine (Zhang & Siegelbaum, 1991). The interesting result is that there is no change in sodium current amplitude of wt HSV 17^ infected DRG neurones after incubation with chloroquine which can be explained by a shift in the current-voltage relationship.

The result of inhibiting internalisation with chloroquine or ammonium chloride suggested that internalisation of sodium channels may be the mechanism for the loss of sodium currents following a wt HSV 17^ infection. Unfortunately, the interpretation of the data obtained using chloroquine and ammonium chloride was not straightforward as these agents have multiple actions on cell functions. The presence of chloroquine and ammonium chloride causes aggregation of endosomes (termed vacuolation) inhibition of exocytosis, as well as endocytosis (Mellman et al. 1986).

Another important action to consider is that these agents inhibit the maturation of wt HSV 17^ by preventing viral envelopment (Koyama & Uchida, 1984). Although chloroquine and ammonium chloride do prevent the loss of sodium conductance associated with wt HSV 17^ infection, it could be argued that this was an effect of vacuolation, or inhibition of viral envelopment, rather than inhibition of internalisation. These possibilities were investigated with experiments using bafilomycin A1 and brefeldin A.

The fungal (Eupenicillum brefeldiaum) toxin Brefeldin A disrupts secretory membrane traffic by inhibiting the formation of Golgi vesicle coat proteins (Donaldson et al. 1992; Helmes, B. & Rothman, E. 1992). Treatment with Brefeldin A results in a rapid collapse of the Golgi apparatus structure which then fuses with the endoplasmic reticulum, whilst the endocytic pathway remains unaffected (Lippincott Schwartz et al. 1989). Envelopment of wt HSV 17^, which occurs along the secretory pathway, is inhibited by treating infected cells with brefeldin A (Koyama & Uchida, 1994). Brefeldin A was used to investigate whether inhibition of viral envelopment could inhibit loss of sodium currents observed in infected DRG neurones. The sodium conductance of wt HSV 17^ infected DRG neurones were still lost when cultures were incubated with brefeldin A. This suggests that the mechanism of sodium current reduction was independent of any Golgi apparatus function and that viral envelopment
was not necessary. Thus these results indicate that the effects of chloroquine and ammonium chloride were not due to their ability to inhibit viral envelopment.

More evidence that channel internalisation was responsible for the loss of sodium currents was obtained from experiments with the Streptomyces antibiotic, bafilomycin A1 (Werner et al. 1984). Bafilomycin A1 is a very specific inhibitor of the V-ATPases, which regulate the pH of endosomes. It does not inhibit F0,F1-ATPases of the mitochondria or E1,E2 ATPases of the plasma membranes (Bowman et al. 1988). The effect of inhibiting the V-ATPases results in the neutralisation of acid endosomes (Yoshimori et al. 1991). When endosomes become neutral, the enzymes involved in membrane budding are inactive, consequently membrane traffic and internalisation are inhibited. Incubation of wt HSV 17+ infected DRG neurones in the presence of bafilomycin A1 prevented the loss of sodium conductance associated with a wt HSV 17+ infection. As Bafilomycin A1 is a specific drug the results can be interpreted more confidently than the experiments with ammonium chloride and chloroquine; inhibition of internalisation prevents the effects of wt HSV 17+ infection on sodium currents. An explanation for this effect is that sodium channels were internalised seen at 24 hours infection with wt HSV 17+ and that sodium channels remained in the plasma membrane when internalisation was blocked.

There is a precedent for internalisation of cell surface proteins associated with wt HSV 17+ infected cells. Studies have demonstrated that a wt HSV 17+ infection of arterial smooth muscle cells results in an increased internalisation of the LDL receptor (Hsu et al. 1995). This internalisation contributes to an accumulation of cholesterol within infected cells. Infection of arterial cells with a HSV-1 deletion mutant lacking the gene U53, encoding for a viral protein kinase, prevents the accumulation of cholesterol. It is tempting to speculate that the increased internalisation of the LDL receptor was stimulated by the viral protein kinase which may affect the internalisation rate of any protein in the endocytic route. The viral protein kinase encoded by U53 phosphorylates the viral membrane phosphoprotein encoded by U134 (Purves et al. 1992). The function of the abundant membrane phosphoprotein encoded by U134 is unknown, although is non-essential for viral replication. The phosphorylation of this
viral membrane protein could result in the stimulation of LDL receptor internalisation. If sodium channels are internalised in the same manner as the LDL receptor, then the viral protein kinase could be involved, directly or indirectly, in the internalisation process of sodium channels. A simple experiment to investigate the role of viral protein kinases in the internalisation of sodium channels, would be to infect DRG neurones with the mutant lacking U₃ gene (Purves et al. 1987) and study effects on sodium current amplitudes. Unfortunately, this virus was unavailable for testing.

The results described in this chapter demonstrate that incubating DRG neurones with a combination of veratridine and KCl results in a loss of sodium conductance. This finding is consistent with a loss of sodium conductance observed in neonatal rat brain cultures which were also incubated in both veratridine and KCl (Dargent et al. 1994). In these experiments, the prolongation of sodium channel open times by sodium channel activator, veratridine, in depolarising media was sufficient to trigger internalisation of the voltage-gated sodium channels from the plasma membrane of these neonatal neurones. The disappearance of sodium channels from the neurone surface was observed either by measuring [³H] saxitoxin specific binding or monitoring changes in [²²Na⁺] uptake (Dargent & Couraud, 1990). The presence of the sodium ionophore amphotericin B also resulted in sodium channel internalisation, suggesting that an increase in intracellular sodium concentration could trigger sodium channel downregulation (Paillart et al. 1996).

DRG neurones treated with both veratridine and KCl, to prolong sodium influx through sodium channels, resulted in a similar loss of sodium conductance which could be explained by internalisation of voltage-gated sodium channels. In contrast there was no corresponding loss of potassium or calcium currents with this treatment.

Control experiments showed that the loss of sodium conductance, seen when DRG neurone cultures were incubated with a combination of 0.1 mM veratridine and 50mM KCl, was a result of prolonged sodium channel opening and not an effect of either of the agents alone. The normalised sodium current amplitudes, measured from DRG neurones that were incubated for 24 hours with 0.1 mM veratridine alone, followed by thorough washing, were unchanged compared to the controls. There was a small, but
not significant, trend for normalised sodium currents from DRG neurones treated with 50mM KCl alone to be smaller than those from control DRG neurones. This could be because the depolarising effects of KCl on the membrane potential of DRG neurones activated some voltage-gated sodium channels. The subsequent influx of sodium ions may have been sufficient to stimulate internalisation of some sodium channels, thus decreasing the sodium current amplitudes slightly. The voltage dependence of the currents was unaffected by the prior incubation with either veratridine or KCl. The effects of treating DRG neurones with a combination of veratridine plus KCl was a result of prolonged channel opening. Preliminary observations of the current-voltage plots and activation curves of sodium currents of DRG neurones treated acutely with 0.1mM veratridine suggested that these were altered compared to the controls. Statistical analysis of the $V_{50}$ and slope factors, however, showed that the voltage dependence of the sodium current activation was not significantly altered. The normalised sodium current amplitude was unaffected by acute addition of veratridine to the bath solution. This finding demonstrates that the presence of any residual veratridine in the bath solution could not account for the loss of sodium conductance observed when DRG neurones were pretreated with both veratridine and KCl.

The loss of sodium conductance associated with wt HSV 17+ infection was prevented when infected DRG neurones were incubated either in the presence of sodium channel blocker, TTX, or in sodium free media. These results suggest that sodium influx was necessary for the loss of sodium conductance in wt HSV 17+ infected DRG neurones. This notion is consistent with the results from studies on neonatal rat brain neurones in which sodium channel down regulation was not observed in neurones treated with a combination of veratridine and KCl, either in the presence of TTX, or in sodium free media (Dargent & Couraud, 1990). Thus, sodium influx was necessary for the internalisation of sodium channels.

The theory that sodium influx and thus the rise of intracellular sodium concentration occurs during a wt HSV 17+ infection could be investigated with a sodium sensitive dye such as SBFI (sodium binding fluorescent indicator). The down regulation of sodium channels after treatment of neonatal rat brain cultures with veratridine and KCl was very rapid ($t_{1/2} = 15$ mins) (Dargent & Couraud, 1990). The rate of sodium
channel loss after wt HSV 17\(^+\) infection is unknown, although there may be a window during wt HSV 17\(^+\) infection when intracellular sodium concentration rises and stimulates the loss of sodium channels. This may occur 1-2 hours before the reduction in sodium currents is observed. Analysis of sodium current amplitudes during the time course of wt HSV 17\(^+\) infection of DRG neurones shows a very sudden reduction in sodium current amplitudes after 20 hours (chapter 3, figure 3.9). This loss is compatible with the idea that sodium channel density could be rapidly reduced following a rise in intracellular sodium concentration. It would be interesting to discover the precise timing of any such change and to determine if the rise in intracellular sodium could be inhibited by TTX. Such information would lend further support to the notion that sodium entry via voltage-gated sodium channels during a wt HSV 17\(^+\) infection triggers the internalisation of sodium channels from the DRG neurone surface.

There was a slight decrease in the normalised sodium currents of DRG neurones treated with TTX compared to those of the control neurones. This could simply be due to the ineffective wash out of TTX. There was also a larger variation in the current amplitudes of wt HSV 17\(^+\) infected, TTX treated DRG neurones, which may also reflect ineffective TTX washout.

The results obtained from DRG neurones incubated in the absence of sodium were difficult to interpret as this treatment alone significantly reduced sodium current amplitudes. As the loss of sodium conductance from wt HSV 17\(^+\) infected DRG neurones occurs between 20 and 24 hours (figure 3.9), it would be interesting to remove sodium from the media at 16 hours post infection to prevent the inherent reduction in sodium current amplitude seen with this treatment. Thus, the neurones would be incubated in the absence of sodium during the critical period of wt HSV 17\(^+\) infection when sodium influx occurs. It would also be interesting to leave the neurones in normal F-14 medium for an hour before electrophysiological recordings were made, in an attempt to rectify any alterations in sodium homeostasis. Although there is a reduction in sodium current amplitude of DRG neurones incubated in sodium free media for 24 hours, there is no comparative loss of sodium current amplitude of wt HSV 17\(^+\) infected DRG neurones also incubated in sodium free media. Furthermore, the percentage of neurones without a sodium current was much higher for the wt HSV
infected DRG neurones incubated in normal, F14 media (87%), than for the wt HSV 17\textsuperscript{+} infected DRG neurones incubated in sodium free media, (25%). 23\% of uninfected neurones also incubated in sodium free media showed no sodium current. This indicates that the loss of sodium currents from DRG neurones incubated in sodium free media may be an effect of the low external sodium rather than an effect of the viral infection.

The most likely explanation for the reduction in sodium current amplitudes in sodium free media is that the absence of external sodium renders the electrogenic pump non-functional. In physiological conditions an increase in internal sodium concentration increases the activity of the pump. When there is no external sodium, however, sodium influx does not occur and intracellular sodium concentration does not rise, so that the pump is never stimulated. Under normal conditions the transport ratio of the pump is 3:2, that is, 3 sodium ions are transported outwards in exchange for 2 potassium ions transported inwards. This results in a net outward current which hyperpolarises the cell. When the pump stops there is an immediate drop in the membrane potential because the hyperpolarising current is removed (Thomas, 1972). Thus, the membrane potential becomes depolarised when sodium ions are removed from the external media. This may be similar to the depolarising effect of 50mM KCl.

The run down of membrane polarisation and unregulated intracellular sodium concentration may be sufficient to cause a decrease in sodium current amplitudes. This could be confirmed by measuring the sodium current amplitudes of DRG neurones following incubation of cells in ouabain which inhibits the Na\textsuperscript{+}/K\textsuperscript{+} ATPase pump, for a 24 hour period.

An interesting in vivo example of increased sodium influx resulting in decreased sodium channel density is found in a mouse model of epilepsy called tottering mouse, in which prolonged neuronal hyperexcitability and spontaneous epilepsy occurs. The hyperexcitability of the central neurones results in an 80\% increase in sodium ion influx via voltage-gated sodium channels as measured by \textsuperscript{22}Na\textsuperscript{+} influx. This activity resulted in a decrease of sodium channel density measured by saxitoxin binding (Willow et al. 1986). Receptors for neurotransmitters are often down regulated after
prolonged agonist exposure which acts as a negative feedback pathway maintaining an appropriate level of responsiveness (Stiles et al. 1984). Similar down regulation of sodium channels after increased electrical activity could play an important role in controlling excitability. The levels of voltage-gated sodium channels in rat myotubules after being treated with sodium channel blockers such as TTX, to prevent spontaneous excitability, were found to be significantly larger than control myotubes (Sherman & Catterall, 1984). This finding again suggests that sodium influx can regulate the density of voltage-gated sodium channels in excitable cells.

In addition, the process of sodium channel internalisation from the surface of neonatal central neurones is resistant to inhibitors of protein kinases (Dargent et al. 1995). This observation makes it unlikely that the viral protein kinase (encoded by Us3) which was implicated in the internalisation of the LDL receptor plays a role in sodium current loss.

The Western blot analysis of sodium channel α subunit protein from either infected or uninfected DRG neurones showed no difference in protein levels. If the loss of sodium channels from the plasma membrane of the DRG neurones after 24 hours of wt HSV 17+ infection were accompanied by protein degradation, then a visible difference in the protein levels of infected and uninfected neurones may have been expected. Any such loss of plasma membrane sodium channels may, however, have been masked by large intracellular pools of sodium channel α subunits, which would also be recognised by the antibody probe. The plasma membrane sodium channel levels could have been investigated by not permeabilising the neurones, such that the antibody bound only to the surface sodium channels. This procedure, however, was not possible in these experiments as the antibody was directed against an internal region of the sodium channel and thus neurone permeabilisation was necessary. Another method of investigating the fate of cell surface proteins is to catalytically bind [125I] to surface proteins using the Bolton-Hunter reagent followed by Western blot analysis. This procedure enables the quantitative detection of [125I] labeled cell surface proteins, which recognise the sodium channel antibody. Alternatively cell proteins could be immuno precipitated with the anti-sodium channel antibody and levels of
radioactivity monitored. This method was not employed, however, due to time constraints.

Visualisation of the intracellular location of sodium channels after 24 hours of wt HSV 17+ infection was achieved by indirect immunofluorescence, with a sodium channel antibody. The smooth staining pattern of the sodium channel seen in control neurones became punctate after 24 hours wt HSV 17+ infection. In contrast the staining pattern for potassium and calcium channels was uniformly diffuse and was identical both before and after infection. The punctate staining pattern of the sodium channels after 24 hours infection with wt HSV 17+ is very similar to that of the lysotracker dye which stains acid endosomes. These results are consistent with the hypothesis that sodium currents are selectively lost from the surface of DRG neurones after a lytic infection with wt HSV 17+, by the selective internalisation of sodium channels into the acid endosomes. The punctate staining of the sodium channels of DRG neurones treated for 5 hours with veratridine and KCl also indicated internalisation. After 24 hours treatment with both veratridine and KCl no sodium channel staining was seen, possibly because the internalised sodium channels had been degraded.

The advantage that a virus may gain by selectively down regulating sodium channels is unknown. The loss of sodium currents may have a specific function in the replication cycle of wt HSV 17+. Viral replication is a highly ordered operation in which genes are tightly regulated in a temporal fashion (Honess & Roizman, 1974). The infection of an already infected cell with another virus would cause chaos in the replication cycle and the production of viral progeny would be inefficient or even non-existent. Furthermore, latent HSV-1 hosted in a sensory neurone would be disrupted by superinfection (Scheck et al. 1989). The viral gene activators such as ICP25 (VP16 or αtif) or ICP4 could activate genes on the latent DNA and initiate transition to the lytic state. Many viruses have developed methods of preventing superinfection of the host cell. A well characterised example of this is the infection of T-lymphocytes with human immunodeficiency virus (HIV). One of the many functions of HIV nef protein is to down regulate CD4 by accelerating its endocytosis. Nef protein also downregulates CD4 expression at the cell surface by retaining the protein in the
endoplasmic reticulum and the Golgi apparatus. Consequently, CD4 is lost from the plasma membranes of T-lymphocytes. As CD4 is the principle virus receptor for HIV attachment, the removal of CD4 from the cell surface prevents superinfection (Little et al. 1994; Layne et al. 1990).

The spread of HSV-1 to sensory neurones after primary infection of epithelial cells may require specific viral attachment to neuronal membranes. Abundant and specific neurone cell membrane proteins are sodium channels. A hypothesis is that HSV-1 attaches to the sodium channels of sensory neurones, followed by adsorption and fast retrograde axonal transport of the virus to the cell body, where viral replication can occur (Topp et al. 1994). If HSV-1 triggered internalisation of sodium channels, subsequent superinfection could not occur. There is no existing evidence in the literature to support this hypothesis, but this could be investigated by removing sodium channels from the surface of DRG neurones by incubation with both veratridine and KCl. Any subsequent reduction in the invasiveness of HSV-1 could then be monitored.

In summary, the results in this chapter have shown that inhibition of plasma membrane internalisation in wt HSV 17^ infected DRG neurones, prevented loss in sodium conductance after 24 hours infection. When sodium influx was prevented either by blocking sodium channels with TTX or by removing sodium from the external media, the reduction of sodium current amplitudes usually observed with a wt HSV 17^ infection did not occur. This result was consistent with studies of neonatal rat brain neurones which provide direct evidence for the internalisation of sodium channels after sodium ion influx. It is thus suggested that during a wt HSV 17^ infection of DRG neurones a sodium influx triggers the internalisation of a sufficient number of sodium channels to result in the loss of a measurable sodium conductance.
Chapter 6

HSV based viral vectors for gene therapy

Introduction

Genetically engineered viruses are increasingly being considered as vectors for the delivery of therapeutic genes into diseased tissues. An ideal viral vector for the study of neuronal mechanisms, or for the gene therapy of neurological diseases, would require efficient gene delivery to postmitotic neurones \textit{in vivo} in addition to a promoter system able to produce persistent and appropriate levels of therapeutic gene expression, without causing cytopathic effects. There are many advantages in the natural biology of HSV-1 which make it an attractive viral vector for gene transfer into the nervous system. Key among these advantages is the fact that the HSV-1 viral genome is a large, 152 kb in length, linear double stranded DNA molecule that
encodes for at least 75 genes (McGeoch et al. 1988). These genes can be divided, roughly in half, into those that are essential for viral growth in cell culture and those that are not. It is possible, therefore, to remove large segments of the viral genome and insert up to 50 kb of foreign DNA into the viral genome (Glorioso et al. 1995). Another important advantage is HSV’s ability to infect postmitotic neurones where it persists in a life-long latent state, without viral protein synthesis, genome integration or interference with host cell biology (Roizman & Sears, 1987). During latency the only transcriptionally active region of the genome maps to a segment of the inverted repeat sequence of the unique long region which encodes the latency-associated transcripts (LATs) (Fraser et al. 1992). There are two latency active promoters (LAP1 and LAP2) for LAT expression during latency that have been characterised upstream of the LAT coding sequence (Goins et al. 1994; Javier et al. 1988). Although Northern blot analysis of the sensory neurones of mice harbouring latent HSV-1 show abundant levels of LAT RNA, so far no LAT-related proteins have been detected during latency (Javier et al. 1988). These promoters could be exploited to drive expression of foreign genes for the gene therapy of postmitotic neurones.

The unique relationship HSV-1 has with neurones provides the potential for efficient, long-term gene transfer to neurons as well as the possibility of long-term foreign gene expression by exploitation of the viral latency active promoters.

HSV-1 replication in neurones, however, has cytopathic effects; wt HSV 17^ infection of DRG neurones resulted in a loss of excitability, due to a loss of sodium conductance after 24 hours (as described in chapter 3). Furthermore, the spread of lytic infections of HSV-1 through neurones of the central nervous system results in fatal encephalitis. It is therefore necessary to design viral vectors in which viral replication is not possible. Deletion of genes essential for viral replication, such as the genes encoding ICP4 or ICP27, is sufficient to prevent cytotoxic effects from occurring (Johnson et al. 1994; Rice & Knipe, 1990; Dobson et al. 1990). Genes necessary for replication specifically in neurones can also be removed to form a disabled virus. The removal of the gene which encodes for protein ICP34.5, which has been defined as the HSV neurovirulence factor, prevents replication in neurones, whilst growth in dividing fibroblasts still occurs (Chou et al. 1990). The structural tegument protein ICP25 (also
known as Vmw65 or VP16), plays a role in the initiation and enhancement of the lytic cycle. In this case ICP25 binds with the cellular transcription factor octomer binding protein 1 (Oct 1) and together these proteins recognise the specific sequence found in the promoters of all the viral immediate early genes (Batterson & Roizman, 1983). ICP25 is not essential for viral replication although it does greatly enhance transcription of the immediate early genes. Mutations resulting in inactivation of the transactivating function of ICP25 can be used to produce a non-pathogenic virus. ICP25 is not fully mutated since it is a structural protein necessary for the assembly of the virion. The mutation, instead, is a small insertion which disrupts its ability to activate immediate early gene expression (Coffin et al. 1996).

Gene transfer with a nonpathogenic HSV-1 derived vector was successfully employed to stably express β-galactosidase in mouse neurones (Dobson et al. 1990). The essential genes encoding for ICP4 were deleted and the β-galactosidase gene under the control of a strong promoter MMLV LTR, (Maloney murine leukaemia virus long terminal repeat promoter) was inserted in the ICP4 gene region. The β-galactosidase gene, was expressed during latent infection of sensory ganglia in vivo. Therapeutically, there are many diverse targets which would benefit from the expression of foreign genes delivered by viral vectors. Some examples of possible foreign gene expression and target tissue are; tyrosine hydroxylase expression in the substantia nigra of Parkinsonian patients to regain secretion of L-DOPA, nerve growth factor (NGF) expression to supply NGF in cases of NGF deprivation following traumatic axon injury such as axotomy, and expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT) in neurones of HPRT deficient rat neuroblastoma cells, which is a model for Lesch-Nyhan syndrome (Palella et al. 1988; Geschwind et al. 1994; During et al. 1994).

The delivery of foreign genes using viral vectors into the nervous tissue must not alter the normal physiology of the target neurones. As wild type HSV infection profoundly alters the electrophysiological characteristics of DRG neurones. It is important to discover whether infection with viral vectors also results in loss of sodium conductance. Loss of excitability in target neurones after gene therapy with a HSV
based vector is not an acceptable side effect. In order to design safe viral vectors it is necessary to understand the mechanism by which sodium conductance is lost during a wild type infection and ensure that this could not happen during the transfer of genetic material with gene therapy. The results in this chapter describe the effects of infecting DRG neurones with a variety of potential gene therapy vectors.

Potential HSV based vectors, with combinations of disabling mutations, that were investigated in this study, are described in the list below.

Virus 1716 had a deletion in both copies of the ICP34.5 gene.

Virus 171627' had a deletion in both copies of the ICP34.5 gene and had the entire ICP27 gene replaced by *Escherichia coli* LacZ gene under the control of the MMLV LTR promoter.

Virus 176427' has an inactivating insertion mutation in the gene encoding ICP25 (also known as Vmw65), deletion in both copies of the ICP34.5 gene and the entire ICP27 gene replaced by *Escherichia coli* LacZ gene under the control of the MMLV LTR promoter.
Results

In order to investigate the electrophysiological characteristics of viral vector infected neurones it was first important to show that 5 p.f.u/neurone of viral vector was sufficient to infect all the DRG neurones in the culture. In this study two of the viral vectors being investigated, 171627- and 176427-, contained a LacZ expression cassette. As described previously (chapter 3) infected neurones were detected by screening for the presence of β-galactosidase. Figure 6.1a and b shows a culture of DRG neurones after 24 hours of infection with 171627- and 176427-, respectively. The neurones containing β-galactosidase turned a deep blue as the β-galactosidase catalysed the breakdown of substrate X-gal. The number of blue neurones were counted and it was found that 5 p.f.u/neurone of virus 171627- and 176427- was sufficient to infect the majority of the neurones in the culture.

The electrophysiological characteristics of DRG neurones infected with replication defective viral vectors derived from wt HSV 17+ were investigated in the experiments described below.

Figure 6.1. A population of DRG neurones infected with either 171627- or 176427-.
DRG neurones were infected with a 171627- or b 176427- for 24 hours and then incubated with X-gal to stain the neurones containing β-galactosidase deep blue.
The neurones infected with HSV based viral vectors grew neurites and remained healthy looking during the 24 hour period in culture. Electrophysiological recordings were made after the neurones were replated to remove neurites. Sodium currents of replated DRG neurones infected with HSV based vectors for 24 hours were analysed. The sodium currents were generated by a step depolarisation to +10mV from a holding potential of -80mV. Representative current recordings from the infected neurones are shown in figure 6.2a. Figure 6.2b shows the mean normalised (for neurone size; nA/pF) sodium current amplitudes generated from DRG neurones infected with viral vectors, 1716, 171627* and 176427*. 100% of the viral vector infected and control DRG neurones had a sodium current, however, in comparison only 29% wt HSV 17^ infected DRG neurones had a sodium current remaining. There was no significant difference between control neurone and wt HSV 17^ based viral vector infected neurone normalised sodium current amplitudes. wt HSV 17^ infected DRG neurone normalised sodium current amplitudes were, however, significantly reduced compared to the currents of viral vector infected DRG neurones and control DRG neurones. The loss of sodium currents associated with a wt HSV 17^ infection of DRG neurones was not observed in DRG neurones infected with HSV based viral vectors.
Figure 6.2. The sodium currents of DRG neurones infected with HSV based viral vectors. a Sodium current traces of control, wt HSV $17^+$ infected and HSV based viral vector infected DRG neurones, evoked by a voltage step from -80mV to +10mV. The leak currents were subtracted. b The normalised sodium current amplitude of DRG neurones infected with HSV-1 based vectors. The graph shows the mean of normalised peak sodium currents of uninfected DRG neurones or neurones infected with wt HSV $17^+$ based vectors. The wt HSV $17^+$ infected DRG neurones had sodium currents which were significantly smaller than the sodium currents of HSV based vector infected or control DRG neurones ($P<0.00005$). The normalised sodium currents of uninfected and HSV based vector infected DRG neurones were not significantly different, $P>0.2$. Statistical differences were examined using the Kruskal-Wallis statistical test followed by the Kolmogorov-Smirnov pairwise comparison.
To investigate whether infection of DRG neurones with potential gene therapy vectors altered the sodium current-voltage relationships, a family of sodium current curves was generated for each neurone with a prepulse to -120mV to remove sodium channel inactivation. Current-voltage relationships were constructed (max current against test potential) for each neurone. The mean normalised current-voltage relationships for virus vector infected cells are displayed in figure 6.3. There was no obvious variation in the sodium current-voltage relationship of wt HSV 17-based vector infected DRG neurones and control DRG neurones.

**Figure 6.3.** The sodium current-voltage relationship of DRG neurones infected with HSV based vectors. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (○) n=44, and 1716 (■) n=24, 171627 (●) n=13, 176427 (□) n=9, infected DRG neurones.

Individual activation curves were also constructed from each current-voltage relationship. The mean normalised sodium activation curve of HSV based vector infected cells and control cells is shown in figure 6.4.
Figure 6.4. Conductance-voltage relationship for sodium channels of DRG neurones after 24 hours infection with HSV based vectors. The relationship between \( \frac{g}{g_{\text{max}}} \) and test potential was determined from the current-voltage relationship shown in figure 6.3, as described in the text. The graph shows the normalised conductance ± s.e.m from control neurones (○) \( n=44 \), 1716 (■) \( n=24 \), 171627− (●) \( n=13 \), 176427− (□) \( n=9 \), infected DRG neurones.

The \( V_{50} \) and slope factors of the conductance plots were measured for each DRG neurone recorded and the mean values are displayed in table 6.1. There was no significant change in the voltage dependence of sodium conductance during an infection of DRG neurones with wt HSV 17\(^{+}\) based vectors as there is no significant difference in the electrophysiological characteristics of the sodium currents generated (\( P>0.1 \), Tukey’s post hoc analysis).
Table 6.1. Electrophysiological characteristics of DRG neurones infected with HSV based vectors. \( V_{50} \) refers to the potential ± s.e.m at which sodium conductance \((g)\) is half of the maximum value \((g_{max})\). The slope factor of the normalised conductance-voltage relationship is a function of the voltage dependence of sodium conductances. \( V_{50} \) and slope factors were calculated from Boltzmann curves fitted between the data points of the conductance-voltage plots of each cell. There was no significant difference between the control and viral vector infected neurones, \( P>0.1 \), (Tukey's post hoc analysis).

Analysis of the total sodium current amplitudes of DRG neurones infected with HSV-1 based viral vectors show that there is no change in the normalised amplitude of sodium currents or a variation in the voltage-dependence of the sodium currents generated.

Analysis of the total sodium currents may not show changes in sodium current subtypes. A more detailed investigation into the effects of infecting DRG neurones with replication defective, virus vectors was therefore undertaken. To demonstrate whether the TTX-S and TTX-R sodium current subtypes were affected by an infection with wt HSV 17\(^+\) based vectors, the sodium currents were electrophysiologically separated into the two subtypes. Briefly, total sodium currents were evoked with depolarising pulses to -20mV for 10 msec from a prepulse of -120mV. After a separate prepulse to -50mV when TTX-S channels were inactivated, the resulting currents generated by a step to -20mV, comprised of the TTX-R component. The TTX-S component was obtained by subtracting the TTX-R component from the total current.
The TTX-S current amplitudes were measured and compared with control neurone current amplitudes, as denoted in figure 6.5. There was no significant difference in the TTX-S current amplitudes of DRG neurones infected with wt HSV 17\textsuperscript{+} based vectors. The potential gene therapy vectors did not alter the normalised TTX-S sodium current amplitudes of DRG neurones.

**Figure 6.5.** The TTX-S normalised mean sodium currents of DRG neurones infected with HSV based vectors. The graph shows the mean normalised TTX-S sodium current ± s.e.m of control DRG neurones or DRG neurones infected with HSV based vectors. There was no significant difference in the mean normalised current amplitudes generated from control or vector infected neurones (analysis of variance followed by Tukey's post hoc analysis, P>0.7).

The current-voltage relationships of TTX-S currents were constructed to investigate the possibility that infection of DRG neurones with viral vectors resulted in a shift in the voltage-dependence of the currents. A family of sodium currents was generated, after a prepulse to -120mV for 200msec, with 10msec command potentials between -80mV and +40mV. TTX-R sodium currents were generated after a prepulse to -50mV for 200msec, with 10msec command potentials between -80mV and +40mV. The
TTX-S current was obtained by subtraction of TTX-R currents from the total currents generated.

The TTX-S currents were normalised with respect to the maximum current and plotted against command potential. The mean normalised peak TTX-S sodium currents from control and virus vector infected DRG neurones are shown in figure 6.6.

![Figure 6.6](image)

*Figure 6.6. The TTX-S sodium current-voltage relationship of DRG neurons infected with HSV based vectors. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (O) n=8, 1716 (■) n=5, 171627' (●) n=6, 176427' (□) n=8 infected DRG neurones.*

The activation curves of the TTX-S sodium conductance were constructed for each neurone tested. The conductance was normalised (g/max) and plotted against command potential. The mean normalised data is shown in figure 6.7.
Figure 6.7. Conductance-voltage relationship for TTX-S sodium channels after infection with HSV based vectors for 24 hours. The graph shows mean normalised conductance ± s.e.m from control neurones (○) n=8, 1716 (■) n=5, 171627− (●) n=6, 176427− (□) n=8. The continuous lines were obtained by fitting a Boltzmann function to the mean normalised data.

The $V_{50}$ and slope factor values of each of the Boltzmann fits were calculated and the mean values are displayed in table 6.2. The statistical analysis demonstrates that there was no alteration in the electrophysiological characteristics of the TTX-S currents of DRG neurones infected with potential gene therapy vectors.

TTX-R sodium currents were generated from control and DRG neurones infected with wt HSV 17+ based vectors, as described previously. The mean normalised TTX-R sodium current amplitudes (nA/pF) are shown in figure 6.8. There was no significant change in the normalised mean TTX-R sodium currents. Infection of DRG neurones for 24 hours with wt HSV 17+ based vectors did not alter the TTX-R sodium current amplitudes.
Figure 6.8. The TTX-R normalised mean sodium currents of DRG neurones infected with HSV based vectors. The graph shows the mean normalised TTX-R sodium current ± s.e.m of control DRG neurones or infected with HSV based vectors. There was no significant difference in the mean normalised current amplitudes generated from control or vector infected neurones (analysis of variance followed by Tukey’s post hoc analysis, P>0.2).

A series of TTX-R sodium currents were generated as described above. The mean normalised TTX-R sodium current-voltage relationships of control and viral vector infected DRG neurones are illustrated in figure 6.9. The current-voltage relationships almost overlay each other. This indicates that there is no alteration in the voltage-dependence of the TTX-R sodium currents evoked from DRG neurones infected with the viral vectors.
Figure 6.9. The TTX-R sodium current-voltage relationship of DRG neurons infected with HSV based vectors. The graph shows the normalised sodium currents ± s.e.m plotted against the command potential from control neurones (○) n=8, 1716 (■) n=5, 171627 (●) n=6, 176427 (□) n=8 infected DRG neurones.

The conductance-voltage relationship of TTX-R currents from wt HSV 17⁺ based vector infected DRG neurones and control DRG neurones is shown in figure 6.10. The electrophysiological values taken from the individual activation curves fitted with a Boltzmann function are presented in table 6.2. The mean electrophysiological characteristics of TTX-R sodium currents from wt HSV 17⁺ based vector infected DRG neurones are not significantly different from the those of control DRG neurones (P>0.7, Tukey’s post hoc analysis). This indicates that there is no change in the TTX-R sodium currents after 24 hours of infection with the viral vectors used in this experiment.
Figure 6.10. Conductance-voltage relationship for TTX-R sodium channels after infection with HSV based vectors for 24 hours. The graph shows mean normalised conductance ± s.e.m from control neurones (○) n=8, 1716 (■) n=5, 171627- (●) n=6, 176427- (□) n=8. The continuous lines were obtained by fitting the mean normalised data to a Boltzmann function.
Table 6.2. Electrophysiological characteristics of DRG neurones infected with HSV vectors. The $V_{50}$ from the TTX-S conductance-voltage relationship is significantly larger than the $V_{50}$ from the TTX-R conductance-voltage relationship, in each case. The slopes factors calculated from TTX-S or TTX-R conductance-voltage relationships were not significantly different from each other. The TTX-R and TTX-S slope factors and $V_{50}$ values from neurones infected with HSV based vectors do not differ significantly from the control values. $V_{50}$ and slope factors were calculated from Boltzmann curves fitted between data points of the conductance-voltage plots of each neurone.

The potential virus vectors investigated were unable to elicit the loss of TTX-S or TTX-R sodium conductance observed after infection with wild type virus. In this respect the wt HSV $17^+$ backbone was successfully disabled such that the loss of normal neuronal physiological function, namely excitability, was not affected. This suggests that these potential HSV based viral vectors could safely deliver therapeutic trans genes to neurones.
Discussion

The potential gene therapy vectors tested here were constructed from the parent virus strain HSV 17+, this strain was also used to characterise the changes in electrophysiological properties of infected DRG neurones described previously. Thus, all the viruses in this study have the same HSV 17+ backbone. This consistency makes direct comparisons between infection with wild type virus and the viral vectors much simpler. Comparison of either total sodium currents or TTX-S and TTX-R sodium currents separately, show that there was no significant difference in the normalised current amplitudes of viral vector infected DRG neurones compared to the control DRG neurones. The infection of DRG neurones with these HSV based viral vectors did not cause the loss of sodium conductance which is usually observed after 24 hours wt HSV 17+ infection. The HSV based viral vector infected DRG neurones were not noticeably different from control neurones in appearance. This is another indication that these potential viral vectors are safe for delivery of therapeutic genes into neurones.

Previous results (chapter 4) have shown that viral DNA replication is necessary for the loss of sodium conductance seen at 24 hours of infection with wt HSV 17+. The viral vectors used were designed to be replication defective by removing essential immediate early genes and/or removal of the neurovirulence factor ICP34.5. The fact that the HSV based viral vectors did not cause a loss of sodium conductance supports the results in chapter 4, that viral replication is necessary for the loss of sodium conductance. This result indicates that these viral vectors are potentially good gene therapy vectors as they do not cause the cytopathic loss of sodium conductance seen with wild type virus infection although the long term deleterious effects in vivo have yet to be excluded. HSV based viral vectors are designed to be avirulent so that delivery of trans genes to the nervous system occurs without cytopathic effects. Any gene which enables the virus to replicate efficiently contributes to the virulence phenotype, and thus any gene which when deleted impairs replication is called a virulence gene. A single gene has
been found in the HSV genome which confers a neurovirulence phenotype: when the gene is deleted the mutant virus has a profoundly decreased ability to cause death in animals. This gene, γ 34.5, maps in the inverted repeats of the UL component and is therefore is present in two copies per genome (Chou et al. 1990). Virus 1716 has both copies of the γ 34.5 gene deleted from parent virus wt HSV 17^+ (MacLean et al. 1991). The virulence phenotype of this mutant reflected a reduced capacity to replicate in neurones whilst growth to high titres could still be achieved in non-neuronal cells. The non-neurovirulence of virus 1716 thus makes it an attractive viral vector candidate. Studies of 1716 expressing a marker gene Lac Z under the control of the latency associated transcript promoter have been used to investigate the performance of the vector in vivo. The results of stereotactic injection of 1716 Lac Z into rat brains were disappointing however, as the expression of trans gene Lac Z was not limited to the site of injection suggesting spread of the virus in non-neuronal cells. In addition, expression did not extend beyond 7 days (McMenamin et al. 1998). The rats showed signs of clinical illness, immune responses and brain inflammation as a result of stereotactic injection of 1716 Lac Z (McMenamin et al. 1998). This suggests that in order for a 1716 to be a successful viral vector other modifications must be made, such as the removal of immediate early genes, so that replication in non-neuronal cells does not occur. The further disablement of 1716 virus was achieved by removal of the essential immediate early gene, ICP27, to produce virus 171627+. When 171627+ was stereotactically injected into rat brains there was less cell damage, and more efficient gene delivery compared to injection of 1716 (Howard et al. 1998). It is advantageous to design a viral vector with more than one deletion, as reversion to wild type virus would require non-homologous recombination with the complementing ICP27 as well as ICP34.5 which would occur very rarely (Howard et al. 1998). Although the ICP27 could be acquired from the complementary cell line, as there is no need to have ICP34.5 gene in the cell line, then there is no chance of the vector acquiring the gene for ICP34.5. Better promoter systems need to be designed that are capable of longer term gene expression. The virus 171627+ may be sufficiently disabled for safe and efficient gene delivery for clinical use.
It is not entirely understood at present how ICP34.5 confers its neurovirulent phenotype. ICP34.5 is thought to prevent the apoptotic premature shutoff of protein synthesis triggered by viral DNA replication which allows continued viral DNA replication in the infected cell (Poon & Roizman, 1997; Chou & Roizman, 1992). The role of inhibiting apoptosis in the neurovirulence phenotype is unclear. For example, there is no evidence illustrating whether or not cells infected with 1716 virus undergo apoptosis and die, but it would be consistent with low gene delivery efficiency. If virally infected cells lacking ICP34.5 do undergo cell death by apoptosis then it could explain the reduced viral spread in the CNS of animals injected with 1716, as the neurones would die before lytic spread was possible. The increase in efficiency of gene delivery to CNS by 171627 may result from the fact that the lack of functional ICP27 prevents viral DNA replication, which in turn prevents the initiation of the apoptotic response and thus prevents cell death.

Another possible mechanism by which ICP34.5 confers the neurovirulence phenotype may result from the fact that ICP34.5 is necessary for maturation and egress from the nuclei (Brown et al. 1994). There is also some debate as to whether ICP34.5 plays a role in the establishment of latency. Different cell types infected with mutant virus lacking the gene encoding ICP34.5 show varying levels of the LATs (latency associated transcripts) which are markers for latently infected cells. In the mouse eye model LATs were not detectable by Northern blot analysis, whereas in the central nervous system expression of LATs was comparable with wild type levels (Spivack et al. 1995; Kesari et al. 1996). This may be an important consideration since viral vectors must efficiently form a latent infection in order for transgene expression to occur in the correct location. It is important to understand the functions of ICP34.5 before designing viral vectors as, although it confers neurovirulence, it maybe an advantage to have a viral vector which prevents apoptosis or loss of latency. Furthermore, in vivo studies of ICP34.5 mutant HSV virus injected into rat and mice brains showed massive inflammation around the site of injection. This suggests that further modifications need to be made to limit the damage caused by the cytotoxic immune responses (McMenamin et al. 1998; McMenamin et al. 1998).
In summary, HSV-1 is promising for use as a vector as its natural biology involves long term persistence of the viral genome in a non-integrated state, specifically in neurones. The potential virus vectors investigated here did not cause the loss of excitability usually associated with a wild type virus infection. These HSV based virus vectors therefore do provide a good basis for the development of safe vectors which have sufficient efficiency in gene delivery to be therapeutically beneficial. More work needs to be done to understand gene expression during latency so that specific and long term promoters can be designed. Perhaps promoters which are responsive to drugs administered by the patients could be exploited for regulation of therapeutic gene expression. Further studies investigating the side effects of gene therapy vectors in vivo as well as cytotoxicity studies in vitro need to be undertaken. Viral vectors based on HSV with the neurovirulence factor ICP34.5 deletion have been shown to cause severe inflammation in the brain (McMenamin et al. 1998). Apart from loss of sodium channels and subsequent loss of excitability, severe inflammation is also an unacceptable side effect of gene therapy.
Chapter 7

General discussion

Summary of findings

The findings of this study have shown that changes in voltage-gated ion channels underlie the altered shape of action potentials and the loss of excitability observed in DRG neurones infected with wt HSV 17+. The calcium channel currents and potassium currents investigated in this study were unchanged by wt HSV 17+ infection. The situation was very different, however, for sodium currents. Both the TTX-S and TTX-R sodium currents were either significantly reduced in amplitude or were not measurable at all after 24 hours of lytic wt HSV 17+ infection. This loss of sodium conductance explains the attenuation of excitability associated with wt HSV 17+.
infection, as an action potential is unlikely to occur without the regenerative increase in sodium conductance.

A loss of sodium conductance underlies the loss of excitability during a HSV infection, but the aspects of viral infection that have caused this change were unknown. The findings of this study have shown that viral entry into the neurone alone was not sufficient to cause these changes, thus revealing that virion proteins did not cause the change upon viral penetration. The special viral feature that suppresses host protein synthesis allowing HSV to take over the neuronal transcription and translation machinery was found not to be responsible for the loss of sodium conductance either. Inhibition of viral DNA synthesis was sufficient to prevent the loss of sodium conductance that was usually associated with the viral infection. This indicated that the aspect of the viral replication cycle responsible for the electrophysiological changes was a late process, perhaps conferred by a late gene.

The cellular processes underlying the loss of sodium conductance during a wt HSV 17^ infection of DRG neurones were unknown. The findings of experiments exploring the possibility that sodium channels were internalised provided some evidence in support of this mechanism. Immunochemical analysis indicated that sodium channel localisation changed from the plasma membrane to cytoplasmic aggregations, although confocal microscopical studies would be necessary to confirm this. Support for the hypothesis was provided by the observation that the loss of sodium conductance could be prevented by the presence of internalisation inhibitors during infection.

HSV is the logical choice as an effective gene therapy vector for neurological disorders. The virus has many key features that make it suitable for gene transfer. The main feature is its ability to remain in neuronal nuclei in a latent state without causing cytotoxic effects. Exploitation of this could result in long term expression of therapeutic genes during latency. Moreover, HSV has a large genome size (152 kb) containing many non-essential genes, that allows the virus to accommodate foreign sequences of \(~35\text{kb}\) (Glorioso et al. 1995; McGeoch et al. 1988). The results described in this study, however, show a loss of sodium conductance after 24 hours of
infection with wild type HSV. This poses an unacceptable side effect of gene therapy. Fortunately, the potential gene therapy vectors investigated in this study did not show the loss of sodium conductance after 24 hours of infection. This was primarily because the HSV based vectors were designed not to replicate by removal of the essential immediate early genes. Viral vectors with disabled ICP25 (trans-activation factor) or deletion of ICP34.5 (neurovirulence factor) were also found not to cause the loss of excitability associated with wild type infections. The gene therapy vectors tested in this study form the basis of safe viral vectors that could be used for delivery of therapeutic genes to diseased neurones.

Abnormal neuronal sensations, post herpetic neuralgia and paralysis

Patients suffering from a primary or recurrent infection of HSV report abnormal sensations around the site of infection. There seem to be two types of abnormality, those sensations relating to the prodrome of infection and neuralgic pains after the vesicles have disappeared, known as post herpetic neuralgia. When HSV is reactivated it replicates in the DRG neurone and the unenveloped capsids are transported back to the site of the original infection by anterograde axonal transport (Holland et al. 1998; Topp et al. 1996; Topp et al. 1994). Once the virus is back at the peripheral tissues it enters the lytic phase of the infection cycle. The cold sore vesicles, symptomatic of HSV infections, are formed during this lytic viral replication phase. Before the appearance of these vesicles, however, people report tingling in addition to loss of touch and pain sensations (Andoh et al. 1995). Anecdotal evidence from people who have eaten the Japanese delicacy Puffer fish (that contains residual small amounts of the neurotoxin TTX despite removal of the toxin containing organs) refer to tingling sensations around their lips that is similar to that of a local anesthetic beginning to work. TTX and anesthetics both block sodium channels resulting in loss of neuronal excitability. It is possible that the parasthesia prior to vesicle formation results from the loss of sodium channels from DRG neurones during viral replication. The loss of
sodium channels could also explain the numbness and hypoalgesia common in the recurrent HSV prodrome.

The area around the vesicle becomes swamped with cells and chemicals involved in the immune and inflammatory response to the lytic HSV infection. For example, prostaglandins that are released in response to the inflammatory actions of bradykinin have been shown to shift the activation curve of TTX-R sodium channels in the hyperpolarising direction resulting in more excitable neurones (England et al. 1996). Topical application of caffeine has recently been shown to prevent the onset of HSV induced hypoalgesia (Shiraki et al. 1998). Caffeine, however, has a plethora of effects on neurones including, antagonism of adenosine (A1) receptors, mobilisation of intracellular calcium or perhaps inhibition of phosphodiesterases. It is unclear what causes the prevention of hypoalgesia. It is also unclear if caffeine interferes with the sodium channel internalisation process.

The phenomenon of post herpetic neuralgia may result from a neuronal over-compensation for the lack of excitability of infected neurones. After the infection becomes latent again the neurones may synthesise an increased number of sodium channels to replenish those lost during infection. The threshold of sodium channel activation could also be shifted, resulting in temporarily over excitable neurones that may take months to retune.

Post herpetic neuralgia could result from an altered phenotype of afferent neurones. Sensory neurones that have been exposed to inflammatory mediators can show an altered phenotype. For example, a larger proportion of the DRG neurone population contain the tachykinin substance P (Neumann et al. 1996) as the Aβ subtype of DRG neurones begin to express detectable levels of substance P which normally is expressed by nociceptive neurones. The release of substance P potentiates glutamatergic transmission via increased sensitivity of post synaptic targets in the dorsal horn. If the population of DRG neurones capable of substance P release increases, then this could contribute to post herpetic neuralgia. The presence of inflammatory mediators during vesicle eruption may induce a switch in phenotype of some sensory neurones resulting in increased pain sensations.
Another explanation of post herpetic neuralgia could be that nerve damage at the site of vesicle formation, effectively axotomising the peripheral nerve ending, alters neuronal function.

The abnormal neuronal sensations associated with HSV infection may be viral strain dependent. Reports of neuronal infections with syncytical strains of HSV and pseudorabies virus (that also forms syncytia) described spontaneous firing of action potentials that are not observed after infections with non-syncytial strains of HSV. The strain used in this study, wt HSV 17+, was a non-syncytial strain of HSV-1. There are no reports in the literature describing a correlation between the strain of HSV and the type or severity of the abnormal neuronal sensations. Chicken pox and shingles, caused by Varicella zoster infections, are known to be very painful with severe post herpetic neuralgia following the disappearance of the vesicles. Varicella zoster also forms syncytial plaques. It is unclear whether the post herpetic neuralgia is linked with the scyncytial phenotype. Patch clamp experiments with Varicella zoster infected DRG neurones are not plausible. This is because Varicella zoster virus infects the respiratory tract, posing a hazard to the experimenter and is very difficult to grow in large quantities. It is interesting to consider whether the internalisation of sodium channels and the subsequent loss of excitability or the syncytial strain dependent spontaneous firing phenomenon, results in post herpetic neuralgia.

Mice and rabbits infected with HSV-1 in the hind paw demonstrated posterior paralysis from which most of the animals recovered (Engel et al. 1997). It is conceivable that the motor neurones also become virally infected and consequently lose their sodium channels via internalisation, just like the sensory neurones. Animals regain functional use of their motor neurones perhaps because once the viral lytic infection has finished they synthesise new voltage-gated sodium channels that allow normal function again. Another incidence of altered motor functions was reported for the Guinea pig jaw opening reflex. The jaw opening reflex of the Guinea pig increased in latency in acutely infected or reactivated infections (Lee et al. 1991). This could be due to a loss of excitability in any of the neurones involved in the reflex, the primary afferent neurones, interneurones or motor neurones. In the light of the results from
this study it is tempting to conclude that the impairment of the reflex stems from the loss of excitability in the primary afferent neurones that are hosting a viral lytic infection. There is much evidence for interneuronal spread within the dorsal horn and HSV antigens can be located in the DRG neurones of adjacent ganglia as the virus works its way up the spinal cord (LaVail et al. 1997). An interesting avenue for further research would be to investigate the electrophysiological changes that occur in different neurone types of the spinal cord.

**Sodium channel phosphorylation, accessory subunits and proteases**

The experimental evidence described in this study suggests that the voltage-gated sodium channels are internalised from the DRG neurone plasma membranes and that this results in the observed loss of excitability. Descriptions of sodium channel down regulation can be found in reference to many neurological situations (see introduction to chapter 5). Another illustration of voltage-gated sodium channel internalisation is found in the survival strategy of neurones in response to anoxic stress (Urenjak & Obrenovitch, 1996). Five minutes after anoxia human brain slices show a markedly reduced excitability (Cummins et al. 1993).

The results described here suggest sodium channels are internalised after 24 hours of wt HSV 17+ infection. Three possible mechanisms that may be involved in the internalisation process are discussed below. Firstly, whether or not viral protein kinases trigger internalisation. Secondly, whether or not virally encoded proteases may be involved in inducing a large influx of sodium ions, and thirdly, the role of sodium channel accessory subunits.

There are two suggestions in the literature that sodium channel phosphorylation may play a role in internalisation. When chromaffin cells were incubated with activators of PKC for 15 hours the number of [3H]STX binding sites were reduced by ~60% (Yanagita et al. 1996). Cyclic AMP dependent protein kinase A activation in rat...
cardiac myocytes was found to down regulate sodium channels by \(~50\%\) in 1 hour. Although, after 2 hours of forskolin treatment the reduction of sodium channels did not exceed 60\%. In spite of the fact that both these results show that the number of sodium channels is reduced after prolonged PKA or PKC stimulation, neither study shows conclusively that the sodium channel proteins were phosphorylated. Instead, phosphorylation of cytoskeleton associated proteins may have effects on sodium channel positioning and stability in the membrane. It is interesting to note that sodium ion influx has been shown to stimulate the breakdown of IP\(_3\) (phosphatidylinositol bisphosphate) that stimulates both the release of calcium from intracellular stores and PKC activation (Gusovsky et al. 1986). Since sodium influx is the trigger for sodium channel internalisation, it is tempting to speculate that PKC could therefore be the second messenger that transduces this signal.

Phosphorylation of sodium channels modulates their function (Cukierman, 1996). Studies with the rat Ila sodium channel either transfected into CHO (Chinese hamster ovary cell line) cells have been used to investigate the role of protein kinase A (PKA). Addition of the PKA catalytic subunit to inside-out patches resulted in \(~40\%\) attenuation of peak sodium currents without any alteration of the kinetic or steady-state kinetics (Gershon et al. 1992; Li et al. 1992). The phosphorylation of serine 573 on the intracellular linker region I-II was responsible for the reduction of sodium currents after PKA phosphorylation (Smith & Goldin, 1997; Smith & Goldin, 1996). Contrary to this, oocytes expressing rat brain sodium channels showed an \(~80\%\) increase of peak sodium currents without any alteration in activation or inactivation when dibutyryl cAMP was added to the pipette (Smith & Goldin, 1992).

Phosphorylation of sodium channels by protein kinase C (PKC) can either enhance or reduce sodium current amplitudes depending on which activator of PKC was used. This indicates that different isoforms of PKC may phosphorylate different residues on the channels that have distinct functional consequences (Renganathan et al. 1995; Numann et al. 1991). Stimulation of PKC with diacylgygyerol (DAG) resulted in a reduction in sodium currents by shifting the steady-state inactivation curve by \(~20\text{mV}\) (Godoy & Cukierman, 1994). In contrast to this, if the PKC was activated with oleic
acid, then the peak sodium currents increased by ~70% (Renganathan et al. 1995). Interestingly, the PKC and PKA signaling pathways seem to converge on the serine residue 1506 of the intracellular linker III-IV region, as phosphorylation by PKC was found to be a prerequisite for the attenuation of sodium currents by activation by PKA (West et al. 1992).

There is some evidence that calcium/calmodulin dependent protein kinase II may be involved in the regulation of sodium channel density. A specific inhibitor of calcium/calmodulin dependent protein kinase II, KN-62, was found to increase the density of sodium channels in chick skeletal muscle (Satoh et al. 1994). Consistent with this KN-62 was found to inhibit the internalisation of sodium channels in the neonatal rat brain (Dargent et al. 1995). These results together suggest that calcium/calmodulin dependent protein kinase II has a role in down regulating sodium channel density in excitable cells. It would be interesting to investigate the role of calcium/calmodulin dependent protein kinase II in the internalisation process during a HSV infection of DRG neurones.

It is not clear whether the reduction of sodium currents by phosphorylation of the channel could be sufficient to explain the loss of sodium currents associated with a HSV infection. If the increased sodium currents corresponding to PKC activation with oleic acid was sufficiently high, then this sodium influx could trigger sodium channel down regulation. In response to these two points, the attenuation of the sodium currents do not seem dramatic enough to account for the total loss of sodium channel conductance seen with HSV infection. The internalisation triggered by activation of PKC and PKA in chromaffin and cardiac myocytes respectively, resulted in an approximately 50% loss of sodium channels. Further experiments with inhibitors of PKC and PKA in infected neurones or veratridine treated neurones may shed more light on this interesting possibility.

The HSV genome encodes for at least two protein kinase genes, U₃·13 and U₃·3 (Roizman & Sears, 1996). The early protein encoded by U₃·3 is located in the cytoplasm (Goshima et al. 1998), and deletion mutants show a decreased
neurovirulence phenotype without affecting replication or establishment of latency (Purves et al. 1987; Meignier et al. 1988). Furthermore, this protein kinase was shown to alter trafficking of membrane proteins in infected arterial cells (Hsu et al. 1995) (see chapter 5). Ul13 is a late protein kinase that is located in the nucleus of infected cells (Roizman & Sears, 1996). It would have been fascinating to investigate the electrophysiological changes of DRG neurones during an infection with either a Ul3 or Ul13 deletion mutant virus, but these mutants were not available.

Serine/threonine phosphatases, phosphatase 2A and calcineurin, have been shown to modulate sodium channel function by dephosphorylation (Kondratyuk & Rossie, 1997). The HSV genome does not encode for any phosphatases, although it is conceivable that a HSV gene product could modulate the normal functioning of neuronal phosphates.

It is well known that the inactivation loop of sodium channels can be cleaved off when proteases are present in the patch pipette which leads to prolonged opening of sodium channels (Hille, 1992). The subsequent sustained activation of sodium channels and influx of sodium ions could trigger the internalisation of sodium channels (Dargent & Couraud, 1990). The HSV genome is known to encode for at least two proteases that are essential for capsid assembly. Could it be that these viral proteases also cleave off the inactivation loop of sodium channels? The subsequent prolonged opening of sodium channels and ion influx could then trigger internalisation of sodium channels. Circumstantial evidence suggests that these proteases may be involved in the process of sodium channel internalisation. The viral proteases are encoded by true late genes and are located in the cytoplasm. This places them in the right place at the right time. Most information is known about the serine protease encoded by Ul26 that cleaves the assembin protein designated ICP35 between alanine and serine residues (Dilanni et al. 1993). Furthermore, a comparison between the Varicella zoster and Herpes simplex proteases found them to be 50% identical and with very similar folding patterns (Qiu et al. 1997; Hoog et al. 1997). The HSV protease has been purified and it would be interesting to perfuse cells with this protease via the patch pipette to investigate whether it could cleave the inactivation loop (Darke et al. 1994). If that was
successful, then infecting DRG neurones with a \(U_{126}\) deletion mutation virus would give an insight into the role of the \(U_{126}\) gene product in the loss of excitability.

What of the assessor subunits of sodium channels? Some evidence suggests that the formation of a complex between the \(\alpha\) subunit and the \(\beta_1\) subunit confers resistance to sodium channel internalisation (Isom et al. 1995; Alcaraz et al. 1997). The evidence stems from the fact that resistance to internalisation changes with postnatal development of rat neurones. There is a strong correlation between sodium channel internalisation and the degree of incorporation of \(\beta_1\) in the sodium channel complex at different postnatal stages. It is possible that the \(\beta_1\) subunit confers some stability to the sodium channels in the plasma membrane by interaction with neuronal cytoskeletal proteins.

It is interesting to consider whether the virus gains a selective advantage by internalising sodium channels. The energy saved by rendering the neurones non-excitable may make viral replication more efficient. If sodium channels are the receptors that HSV attaches to prior to sensory neurone penetration, then the role of internalisation may be to prevent superinfection. The internalisation, however, may simply be a neuronal response to stressful situations as with anoxia described above.

**Na\(^+\)/K\(^+\) pumps**

In the absence of functional Na\(^+\)/K\(^+\) pumps the resting membrane potential of neurones is not maintained. There was no evidence from these studies that the Na\(^+\)/K\(^+\) pumps are affected during a HSV infection. When the resting membrane potential of infected DRG neurones was measured, the potentials were found to be similar to those of the uninfected neurones. One report in the literature showed that Na\(^+\)/K\(^+\) pump activity steadily declined to \(\sim 50\%\) during a HSV infection of a suspension of HeLa S3 cells (Palu et al. 1994). Whether the same decline in Na\(^+\)/K\(^+\) pump activity is true of DRG neurones is unknown.
Cytoskeletal involvement

The fungal toxin cytochalasin D disrupts actin cytoskeleton by depolymerising actin filaments (Goddette & Frieden, 1986). Treatment of cardiac myocytes with cytochalasin D for 1 hour resulted in a reduction in the, rate of rise of the action potential, overshoot and conduction velocity (Lieberman et al. 1973). The myocyte aggregates desynchronised and eventually stopped spontaneous contractions due to a loss of excitability. There is a striking similarity between the results of treating cardiac myocytes with cytochalasin D and infecting then with HSV as described in the introduction (Batra et al. 1976). DRG neurones treated with cytochalasin D for 24 hours again had action potentials that had both a reduced rate of rise and overshoot. After 48 hours in culture the DRG neurones lost excitability (Fukuda et al. 1981). This result is comparable with the result of infecting DRG neurones with HSV for 24 hours. On the basis of the similar electrophysiological effects of infecting excitable cells with HSV and disrupting their actin cytoskeleton, it is worth considering what role actin may play in the loss of excitability resulting from a HSV infection.

Three types of cytoskeletal associated proteins have been copurified with voltage-gated sodium channels, ankyrin, spectrin and syntrophin. Ankyrin₆ has been immuno-localised with sodium channels that are either clustered at the neuromuscular junction or the nodes of Ranvier (Zhou et al. 1998). The presence of ankyrin₆ is required for sodium channel clustering and is thought to play a role in channel localisation. β-spectrin (a member of the dystrophin family) has also been immuno-localised with sodium channels and has both a syntrophin and an ankyrin binding site (Wood & Slater, 1998, Gee et al. 1998). The syntrophin proteins contain specific domains that recognise the C-terminus of sodium channels (Schultz et al. 1998). These specific domains are known as PDZ domains. They are conserved in mammals in addition to plants and bacteria, suggesting that they have a fundamental role in biology.
In summary, the clustering and location of sodium channels involves at least three types of protein. The sodium channel binds via the PDZ domain to a syntrophin protein that also binds to the long and flexible β-spectrin which binds to ankyrin that joins this complex to the actin cytoskeleton. A schematic representation of this arrangement is shown in figure 7.1.

Figure 7.1. The putative arrangement of actin cytoskeleton associated proteins and voltage-gated sodium channels. The actin cytoskeleton associated proteins form a complex that attaches sodium channels to the cytoskeleton. More cytoskeleton associated proteins will probably be isolated in the future.

It would be fascinating to investigate the role of cytoskeletal proteins in the loss of excitability during a HSV infection of DRG neurones. The fate of actin cytoskeleton is unknown in HSV infected cells, although the microtubule network is known to be disrupted (Avitabile et al. 1995). During sodium influx, a high sodium ion concentration will arise around the channels' local environment. This may disrupt the cytoskeleton protein complex which would destabilise sodium channels. There is some evidence that actin monomers, which are the product of cytochalasin D destabilisers, can affect ion channel properties. Epithelial sodium channels were activated when actin monomers, but not polymers, were present in the patch pipette. It
would be interesting to investigate whether actin cytoskeleton stabilizers, such as phalloidin, could prevent loss of sodium conductance from HSV infected DRG neurones, and furthermore, to investigate the effect of actin monomers on the voltage-gated sodium channels of DRG neurones.

In conclusion, a lytic infection of DRG neurones with a non-syncytial strain of HSV-1 resulted in a loss of excitability due to the selective loss of sodium conductance. A late protein or process caused the loss of excitability. This loss of excitability was not, however, dependent on viral envelopment or maturation. The data presented here suggested that sodium channels were lost from the plasma membrane of DRG neurones via an internalisation pathway. A reduction of sodium channels from the plasma membrane could certainly account for the decreased excitability without any shift in the sodium current activation curves, that occurs during a HSV-1 infection. DRG neurones from the adult rat, unlike brain neurones from the adult rat, showed rapid sodium channel internalisation after prolonged sodium ion influx. This sodium channel internalisation triggered by sodium ion influx was investigated as a possible mechanism for sodium conductance loss associated with HSV-1 infections. It was demonstrated that sodium ion influx could be the trigger for sodium channel internalisation during an HSV-1 infection. The possible involvement of the cytoskeleton, viral protein kinases or proteases would be fascinating to study, to further understand the mechanism by which DRG neurones lose excitability during an infection with HSV-1. Finally, the data described here have shown that an HSV infection has dramatic cytopathic effects on neurones that would pose unacceptable side effects for gene therapy. The experiments here have shown that if viral vectors are disabled such that they are replication defective, then the cytopathic loss of excitability does not occur. HSV based viral vectors can be used to transfer foreign genes into neurones without altering the excitability of the target neurone although the long term deleterious effects in vivo have yet to be excluded.
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