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Changes in Excitability Induced by Herpes Simplex Viruses in Rat Dorsal Root Ganglion Neurons

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The physiological properties of rat sensory neurons infected with herpes simplex type 1 viruses and maintained in cell culture were studied using intracellular recording techniques. Two syncytial (cell fusing) and two nonsyncytial strains of virus were examined; individual strains of virus had different effects on neuronal excitability. The nonsyncytial viruses caused a loss of tetrodotoxin-sensitive low-threshold action potentials and blocked hyperpolarization-activated inward rectification, but did not alter the resting membrane potential, depolarization-activated outward rectification, or render the cells leaky. These effects develop progressively over the period 5-15 hr postinfection. One syncytial strain of virus induced spontaneous electrical activity that appeared to be the result of discrete electrical coupling between sensory neuron processes; as a result, action potential discharge is synchronized in coupled neurons. A second syncytial strain of virus rendered neurons inexcitable; however, in these experiments the input resistance fell to low values, possibly as a result of extensive coupling between sensory neurons. Viral replication in sensory neurons was demonstrable with indirect immunofluorescence using an antibody to herpes simplex viruses and correlated with the onset of virally induced changes in excitability. Virally triggered changes in excitability were blocked by the specific herpes virus antimetabolite acyclovir, suggesting that viral adsorption and penetration are by themselves insufficient to evoke changes in excitability. These results suggest that herpes viruses have selective effects on the excitable mechanisms in sensory neurons that are not simply the result of a general loss of membrane conductances or the disruption of transmembrane ion gradients.

Herpes viruses are enveloped particles approximately 100 nm in diameter, have DNA as their genetic material, and bud through the inner nuclear membrane of the infected cell. In a number of vertebrate species, including man, such viruses infect neural tissue and, in particular, sensory ganglion neurons. Since virus-specific antigens and virus-coded glycoproteins are available for insertion into the plasma membrane soon after infection (Norrild et al., 1978; Roane and Roizman, 1964; Spear et al., 1970), it is of interest to examine changes in neuronal membrane function resulting from the infection of nerve cells. Early physiological experiments suggested loss or reversal of membrane potential in epithelial and secretory cells infected with herpes viruses (Fritz and Nahmias, 1972; Fritz et al., 1974; Van Horn et al., 1970; Weigel et al., 1981). However, subsequent experiments on dorsal root and sympathetic ganglion neurons did not reveal changes in resting membrane potential following infection with herpes viruses (Fukuda and Kurata, 1981; Kiraly and Dolivo, 1982; Oakes et al., 1981), despite confirmation of adequate infection by immunofluorescent techniques (Fukuda and Kurata, 1981).

Recent physiological experiments with herpes viruses have described two contrasting effects of infection on the membrane physiology of excitable cells. Herpes simplex virus types 1 and 2 suppress the spontaneous beating of embryonic chick heart cells (Batra et al., 1976) and cause a progressive fall in the rate of rise of the action potential with time following infection, leading to a complete loss of excitability (Schrier et al., 1978). A similar loss of excitability also occurs in dorsal root ganglion neurons infected with these viruses (Fukuda and Kurata, 1981; Gitelson et al., 1978; Oakes et al., 1981). In contrast, sympathetic ganglia infected with pseudorabies virus (herpes virus suis) develop spontaneous electrical activity (Dempsher et al., 1955; Kiraly and Dolivo, 1982). These opposing effects of pseudorabies and herpes simplex viruses on electrical excitability appeared to be virus specific. However, recent studies by Lima et al. (1983) and in our laboratory (Mayer et al., 1985) reported induction of spontaneous electrical activity in dorsal root ganglion neurons following infection with herpes simplex virus types 1 and 2.

The occurrence of strain variations in herpes simplex viruses is well documented (Buchman et al., 1978; Nahmias and Dowdle, 1968; Nii et al., 1983) and is thus a possible explanation for the different effects of individual strains of herpes simplex on neuronal excitability. To address this issue and to better characterize changes in membrane physiology occurring on infection of nerve cells, we have examined the physiological effects of four strains of herpes simplex virus type 1 (HSV1) using cultures of rat dorsal root ganglion neurons. Each of the virus strains was characterized by plaque assay in cultures of BHK-21 cells as syncytial (cell fusing) or nonsyncytial. Infection with either of two nonsyncytial strains of HSV1 caused a loss of excitability; in contrast, another, syncytial, strain of HSV1 induced spontaneous activity that probably results from electrical coupling between nerve cell processes. Abstracts of this work have been presented to the Society for Neuroscience (Mayer and James, 1984) and the Physiological Society (James and Mayer, 1984).

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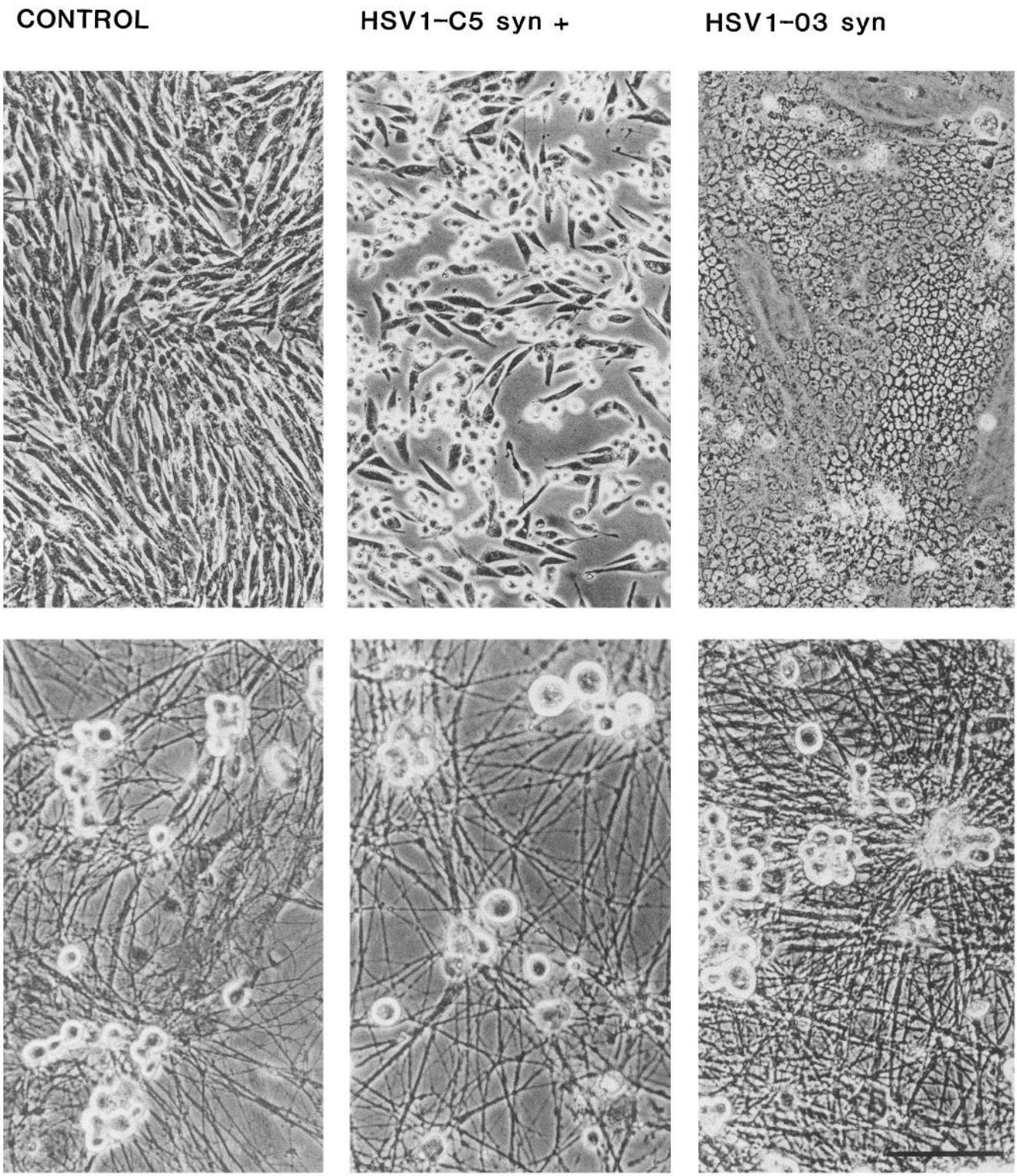

Figure 1. Morphological changes induced by infection with syncytial and nonsyncytial strains of HSV type 1. The *upper row* of phase-contrast photomicrographs shows living BHK-21 cells; the *lower row,* living cultures of rat dorsal root ganglion neurons. Cultures were photographed 16 hr postinfection. Control cultures of BHK-21 cells are phase-dark and confluent. HSV1-C5 syn+ causes rounding of BHK-21 cells, which appear phase bright, but no cell fusion is apparent; in contrast, the syncytial strain HSV1-03 syn produces large plaques of fused cells. Sensory neuron cultures do not show dramatic morphological changes on infection with either strain of virus. Scale bar: *upper row,* 170 μm; *lower row,* 100 μm.

Materials and Methods

Tissue culture

Dorsal root ganglia were removed from 2 d old Sprague-Dawley rat pups and incubated in calcium—magnesium-free Dulbecco's PBS containing 2.5% wt/vol trypsin prior to dissociation by trituration in Dulbecco's PBS containing 1% wt/vol DNase and subsequent plating and growth in Ham's F-12 medium supplemented with 10% fetal calf serum and 5 ng/ml 7s NGF at a density of 30,000 cells/cm^2. Cultures were maintained in a humidified incubator at 37°C with a 5% CO_2, 95% air–

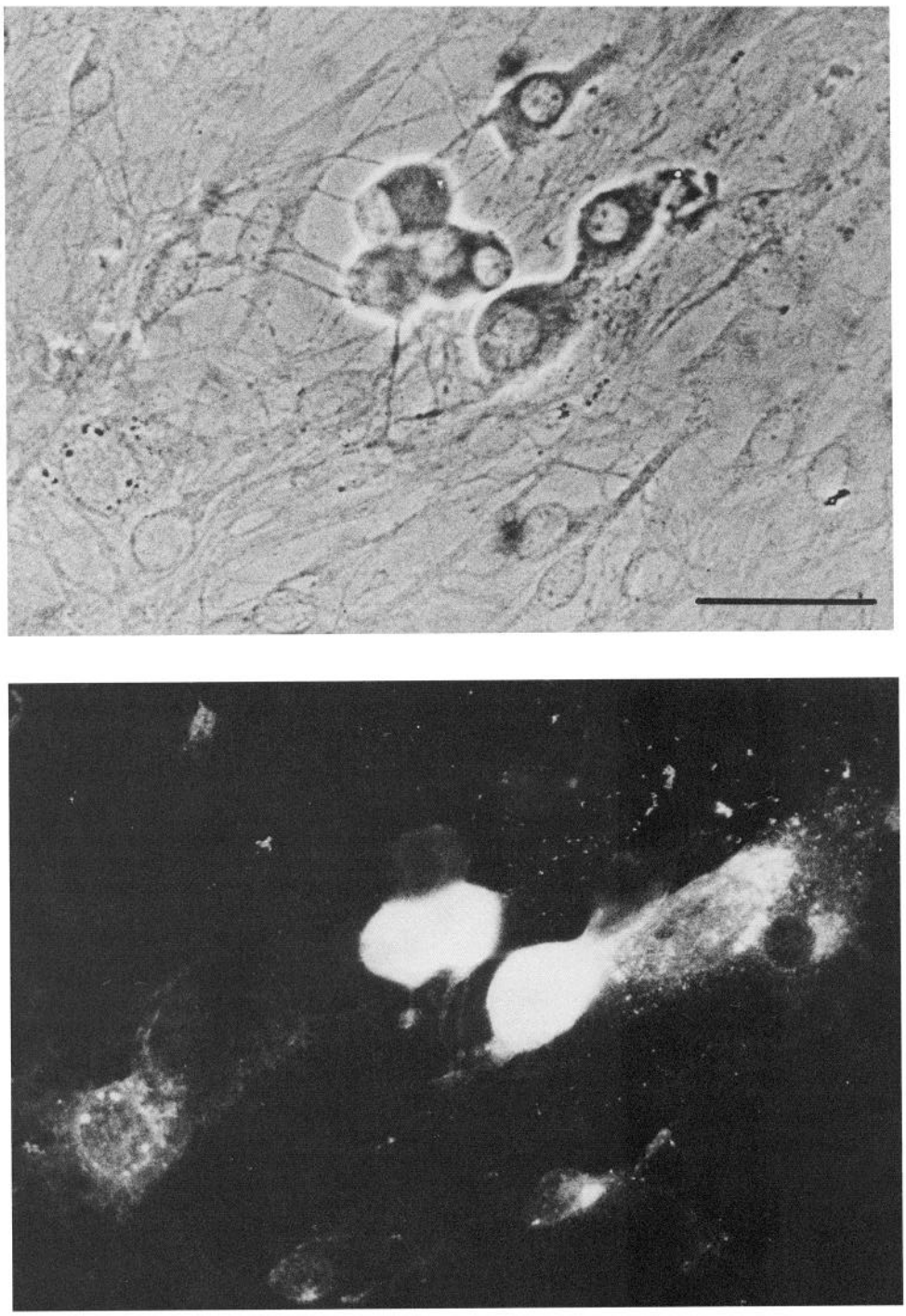

Figure 2. Viral replication in rat sensory neurons in culture. The plates show phase-contrast (*top*) and fluorescence (*bottom*) micrographs of the same field in a culture infected with the syncytial strain HSV1-03 syn. The methanol-fixation procedure used for the immunofluorescent detection of HSV abolishes the phase-bright appearance characteristic of living sensory neurons, but it is still possible to distinguish sensory neuron cell bodies from nonneuronal cells. Three of the seven sensory neurons in the field are strongly immunoreactive for HSV; in addition, two large flat nonneuronal cells show immunoreactivity that is primarily intranuclear. Such viral immunoreactivity was first detectable about 7 hr postinfection with HSV1-03 syn (see Fig. 8), indicating that viral replication in culture is required to achieve sufficient intracellular viral antigen synthesis for detection by immunofluorescence. Scale bar, 50 μm.

gas mixture. At 3–4 d after plating, cultures were exposed to the antimitotic cytosine arabinoside (10 μg/ml) for a period of 48 hr, at which time nonneuronal cells had grown to confluency. Subsequently, the growth medium was changed twice weekly.

Preparation and assay of virus stocks

Virus strains HSV1-C5 syn+ and HSV1-03 syn were gifts from Dr. J. Booth (Department of Virology, St. George's Hospital Medical School, London). In this paper, the standard virological terminology, syn and syn+, is adhered to, where syn refers to *syncytial* and syn+ to *nonsyncytial* strains of virus as classified by plaque morphology assays. Virus stocks were prepared by infecting roller flasks containing confluent monolayers of BHK-21 (HSV1-C5 syn+) or MRC-5 (HSV1-03 syn) cells with virus at a concentration of 0.005 plaque-forming units (pfu) per cell; after incubation at 37°C for 3–4 d, the cells were frozen and thawed, the debris removed by centrifugation, and the resulting supernatant (containing 9–50 $\times$ 10^7 pfu/ml HSV1-C5 syn+ and 5–8 $\times$ 10^7 pfu/ml HSV1-03 syn) stored at -70°C. HSV1-17 syn and HSV1-17 syn+ were obtained from Drs. H. Marsden and J. H. Subak-Sharpe (Institute of Virology, University of Glasgow). Plaque assays, using growth medium containing carboxymethyl cellulose, were used to estimate viral infectivity in 10-fold serial dilutions of viral stock solutions. Experimental infection of neuronal cultures was performed 2–4 weeks after plating at a multiplicity of infection of 20 pfu/cell, using a 1 hr incubation at 37°C, followed by two changes with growth medium containing 10% serum to remove unadsorbed virus; the infected cultures were then incubated at 37°C for up to 48 hr before electrophysiological experiments were performed.

Indirect immunofluorescence microscopy was performed on methanol-fixed cultures using rabbit herpes simplex antiserum and FITC-

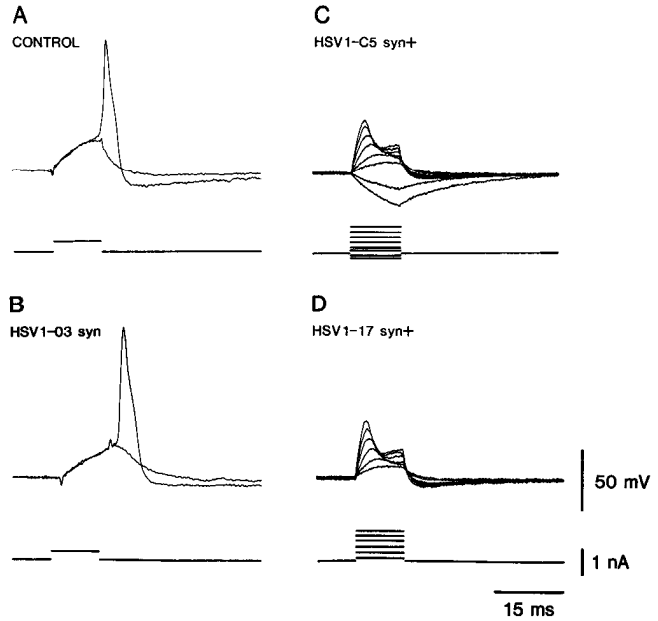

Figure 3. Loss of excitability in cultures infected with the nonsyncytial strains of HSV type 1. *A* and *B*, Two superimposed traces, on threshold, of action potentials evoked by depolarizing electrotonic potentials (*upper traces*), triggered by step current injections (*lower traces*). Data in *A* were obtained from a sensory neuron in a control culture, while those in *B* were obtained from a culture infected with the syncytial strain HSV1-03 syn; *C* and *D* were obtained from neurons in cultures infected with the nonsyncytial strains HSV1-C5 syn+ and HSV1-17 syn+; in each example, a family of depolarizing current pulses, much larger than those required to evoke action potentials in *A* and *B*, fails to trigger regenerative responses. Interruption of the charging transient by outward rectification is obvious in *C* on comparing the time course of the hyperpolarizing and depolarizing electrotonic potentials.

labeled sheep anti-rabbit immunoglobulin (Wellcome Diagnostics RP16 and NF02).

Electrophysiology

Experiments were performed at room temperature (24–26°C) on the stage of an inverted phase-contrast microscope. The recording medium contained (in mM) 141 NaCl, 4.8 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, adjusted pH 7.3. Intracellular recording was performed using either microelectrodes filled with 1 M potassium acetate or patch electrodes for whole-cell recording. The patch electrodes were filled with a solution containing (in mM) 140 KCl, 2 $MgCl_2$, 1.1 EGTA, and 10 HEPES, pH 7.2. An Axon Instruments Axoclamp-2 amplifier, operating either in bridge or discontinuous current clamp modes (switching frequency approximately 10 kHz), was used to record membrane potential and inject current steps. Data was stored on tape, digitized off-line, and plotted on a Hewlett-Packard 770T digital plotter using a PDP 11/23 computer and software developed by J. C. M. Wise.

Results

Morphological changes in infected cultures

Cultures of BHK-21 cells showed characteristic morphological changes on infection with herpes viruses. The nonsyncytial strains HSV1-C5 syn+ and HSV1-17 syn+ produced rounding of cells such that the cultures, which prior to infection were composed of confluent monolayers of flat phase-dark cells, subsequently became phase-bright and were no longer confluent (Fig. 1). The syncytial strains HSV1-03 syn and HSV1-17 syn produced cell fusion and the appearance of large plaques or polykaryocytes (Fig. 1). Similar observations have been made with other strains of HSV1 and have been used to classify herpes viruses into subgroups based on the expression or absence of the fusion reaction (Ejercito et al., 1968; Nii et al., 1983).

Cultures of sensory neurons infected with these same strains of viruses did not show such dramatic morphological changes under phase-contrast microscopy (Fig. 1). Strains HSV1-C5 syn+ and HSV1-17 syn+ appeared to reduce the density of nonneuronal "background" cells, but the general appearance of sensory

Figure 4. Electrotonic potentials evoked by current steps 500 msec in duration reveal loss of inward but not outward rectification in cultures infected with the nonsyncytial strains HSV1-C5 syn+ and HSV1-17 syn+. Each record shows a family of electrotonic potentials (*upper traces*) evoked by step current pulses (*lower traces*). *A*, Typical response recorded from a control sensory neuron. *B*, Similar responses recorded from a sensory neuron in a culture infected with the syncytial strain HSV1-03 syn; in addition, individual traces show spontaneous subthreshold depolarizing potentials and action potentials characteristic of infection with this strain. *C* and *D*, Families of electrotonic potentials recorded from neurons infected with the nonsyncytial strains HSV1-C5 syn+ and HSV1-17 syn+. *E*, Family of electrotonic potentials recorded from a sensory neuron in a culture infected with the syncytial strain HSV1-17 syn; the cell had a fast time constant, low input resistance, and over the range of currents studied, exhibited only passive behavior. All records were obtained under discontinuous current clamp using patch pipettes for whole-cell recording.

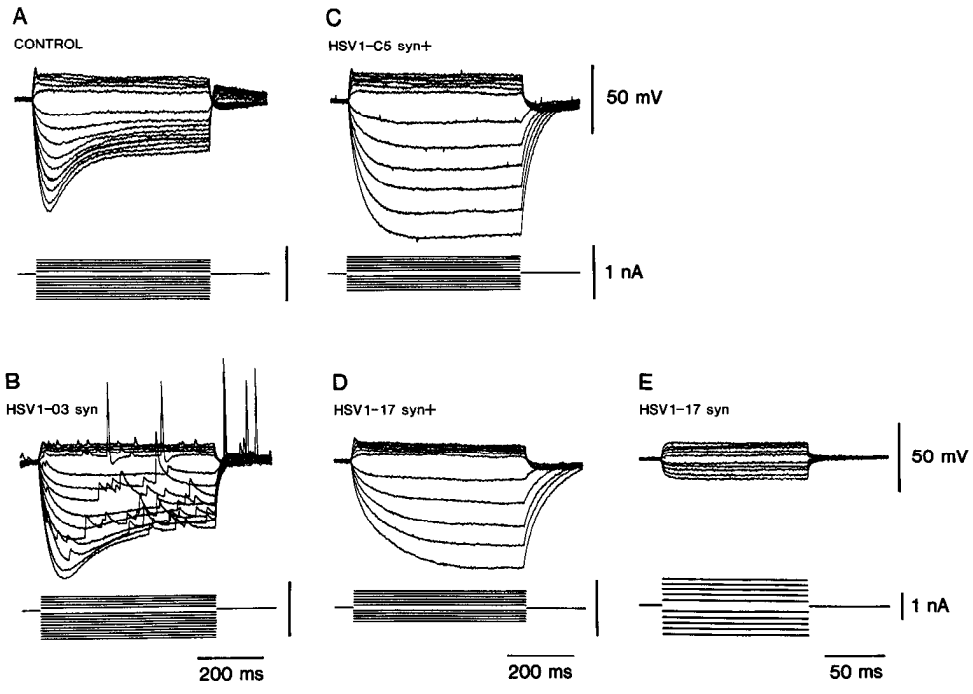

Table 1. Resting potential, input resistance at rest (−60 mV), and action potential amplitude of sensory neurons 15-24 hr after infection with each of the four strains of HSV1 examined

	Control	HSV1-03 syn	HSV1-17 syn	HSV1-C5 syn+	HSV1-17 syn+
Resting potential (in mV)	−57.2 ± 1.3	−58.5 ± 1.6	−58.8 ± 0.4	−53.8 ± 1.4	−54.0 ± 1.4
Slope resistance at −60 mV (in MΩ)	82.5 ± 41	97.2 ± 14	18.1 ± 2	188 ± 34	127 ± 48
Action potential overshoot (in mV)	+38.5 ± 1.3	+35.0 ± 3.7	–	–	–

Values are presented as means ± SEM from observations made on 5–20 neurons. Cells infected with strains 17 syn, C5 syn+, and 17 syn+ were unexcitable, and no values for action potential overshoots are given.

neuron cell bodies, and the network of neurites in mature cultures, was not obviously different in control and infected cultures 15–24 hr after inoculation with virus (Fig. 1). The syncytial strains HSV1-03 syn and HSV1-17 syn produced no obvious changes in sensory neuron morphology (Fig. 1).

Virus replication occurs in sensory neurons and "background" cells

Sensory neuron cultures used for electrophysiological experiments were fixed and processed for microscopy using indirect immunofluorescence to reveal the presence of herpes virus. Figure 2 shows phase and fluoresence micrographs of the same field from a culture fixed 16 hr postinfection with HSV1-03 syn; at this time not all cells were infected, and it was easy to distinguish the infected sensory neurons from adjacent nonfluorescent cells. In the field shown, three out of the seven sensory neurons in the field display strong immunoreactivity for herpes virus, in addition to two large flat nonneuronal cells. Initially, replication of HSV1-03 syn appeared to occur preferentially in such nonneuronal cells and was not detectable until around 7 hr postinfection; however, by 27 hr postinfection, the majority of sensory neurons were also immunoreactive for herpes virus (see Fig. 8). Strain HSV1-C5 syn+ appeared to replicate more rapidly and preferentially in sensory neurons and was detectable 5 hr postinfection; by 12.5 hr, the majority of sensory neurons were strongly immunoreactive for herpes virus.

Electrophysiological studies

Changes in excitability

The majority of electrophysiological experiments were performed 15–24 hr postinfection, at which time the resting potential of sensory neurons in virus infected cultures was similar to that recorded in control uninfected cultures (Table 1). Strains HSV1-C5 syn+ and HSV1-17 syn+, nonsyncytial viruses, caused a loss of excitability similar to that produced by the sodium channel blocker tetrodotoxin (1 μM, TTX) in experiments on uninfected cultures. As a consequence, in the absence of a low-threshold spike-generating mechanism, large depolarizing current pulses activate outward rectification and produce a hyperpolarizing sag in current evoked electrotonic potentials (Fig. 3).

The excitability of sensory neurons in cultures infected with the syncytial virus HSV1-03 syn was normal (see Fig. 3), depolarizing current pulses evoking action potentials with thresholds of −30.0 ± 3.8 mV (control cells, −34.3 ± 2.1 mV) and overshoots of 35.0 ± 3.7 mV (control cells, 38.5 ± 1.3 mV). Despite the apparently normal physiology of the spike-generating mechanism in dorsal root ganglion neurons infected with HSV1-03 syn, these cultures developed spontaneous activity, probably due to virus-induced electrical coupling between axonal processes (Mayer et al., 1985). Such spontaneous activity consisted of subthreshold EPSP-like events that triggered action potentials on crossing threshold (see Figs. 4*B* and 11).

Sensory neurons in cultures infected with HSV1-17 syn, another syncytial strain of virus, were unexcitable. However, in these cultures the neuronal input resistance was considerably lower than that recorded in control cells (see Table 1), and it was usually not possible to sufficiently depolarize the membrane potential of HSV1-17 syn infected cells to reach the threshold for action potentials in control cells.

Inward and outward rectification

Sensory neurons show both inward and outward rectification (Mayer and Westbrook, 1983), and under current clamp the activation of these conductance mechanisms causes time- and voltage-dependent sags in electrotonic potentials evoked by step current injections (Ito, 1957). In the present experiments, step current pulses 120 or 500 msec in duration were used to examine the behavior of sensory neurons at membrane potentials subthreshold for the generation of action potentials. In control, uninfected cells, hyperpolarizing electrotonic potentials show slow depolarizing sags due to activation of inward (anomalous) rectification (Fig. 4*A*). Such rectification also occurs in cultures infected with the syncytial strain HSV1-03 syn (Fig. 4*B*). In contrast, inward rectification is absent in cultures infected with the nonsyncytial strains of virus HSV1-C5 syn+ and HSV1-17 syn+; consequently, the membrane time constant became much longer (Fig. 4, *C* and *D*). Sensory neurons in cultures infected with HSV1-17 syn had low input resistances (Fig. 4*E*); as a consequence, it was difficult to inject sufficient current to examine the rectifying properties of neurons in these cultures.

Depolarizing current pulses subthreshold for generation of action potentials activate a fast outward rectifying mechanism (see Fig. 3). This process occurs in cultures infected with the nonsyncytial strains HSV1-C5 syn+ and HSV1-17 syn+, despite the loss of hyperpolarization-activated inward rectification (Fig. 4, *C* and *D*), and is similar to that recorded in control cultures in the presence of TTX and in cultures infected with the syncytial strain HSV1-03 syn (Fig. 4*B*). In cultures infected with the other syncytial strain, HSV1-17 syn, the low input resistance of the neurons prevented study of outward rectification.

In an attempt to quantify results of the type shown in Figure 4, current–voltage plots were constructed, and slope resistance measurements made at membrane potentials of −40 and −80 mV (Fig. 5). The dramatic effect on inward but not outward rectification produced by infection with the nonsyncytial strains HSV1-C5 syn+ and, to a lesser extent, HSV1-17 syn+ is clearly shown. In contrast, no significant changes were observed between control cultures and those infected with HSV1-03 syn.

Time course of virus action

As we have presented above, 15–24 hr postinfection HSV1-C5 syn+ had produced a complete block of TTX-sensitive action potentials and inward rectification, and HSV1-03 syn had induced spontaneous activity in the majority of sensory neurons.

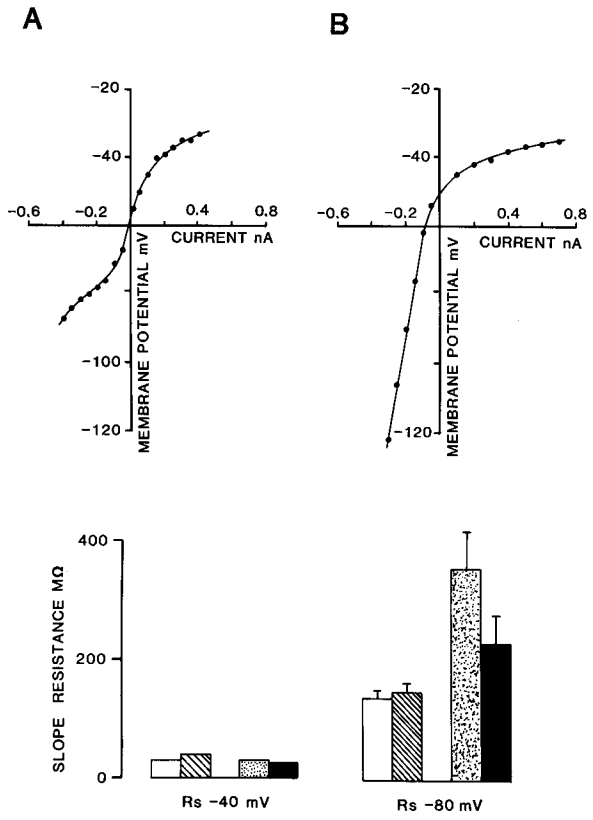

Figure 5. Current–voltage plots obtained from families of electrotonic potentials similar to those shown in Figure 4; slope resistance measurements were made at membrane potentials of −40 and −80 mV by drawing tangents to the current–voltage plots. *A*, Sigmoidal current–voltage relationship characteristic of sensory neurons recorded from a neuron in a culture infected with the syncytial strain HSV1-03 syn; similar current–voltage plots were obtained in control experiments on uninfected cultures. *B*, Current–voltage plot recorded from a neuron in a culture infected with the nonsyncytial strain HSV1-C5 syn+; the slope resistance is constant over the membrane potential range −60 to −120 mV, reflecting loss of hyperpolarization-activated inward rectification. Similar current–voltage plots were recorded in cultures infected with the other nonsyncytial strain, HSV1-17 syn+. The series of histograms shown below summarize slope resistance measurements made at membrane potentials of −40 and −80 mV from control cultures (□) and cultures infected with each of the strains of virus examined in this study: HSV1-03 syn (▨); HSV1-C5 syn+ (▩), and HSV1-17 syn+ (■). Measurements were made from 5–12 neurons and are presented as the means; *error bars*, 1 SEM. At −40 mV no differences are detectable between controls and cultures infected with any of the viruses except HSV1-17 syn, for which sufficient current was not injected to achieve a membrane potential of −40 mV. At −80 mV, the increase in input resistance occurring on infection with both nonsyncytial strains of virus is statistically significant ($p < 0.01$) compared to control values, whereas strain HSV1-03 syn is not significantly different from control. Sufficient current was not injected in cells infected with strain HSV1-17 syn to achieve a membrane potential of −80 mV.

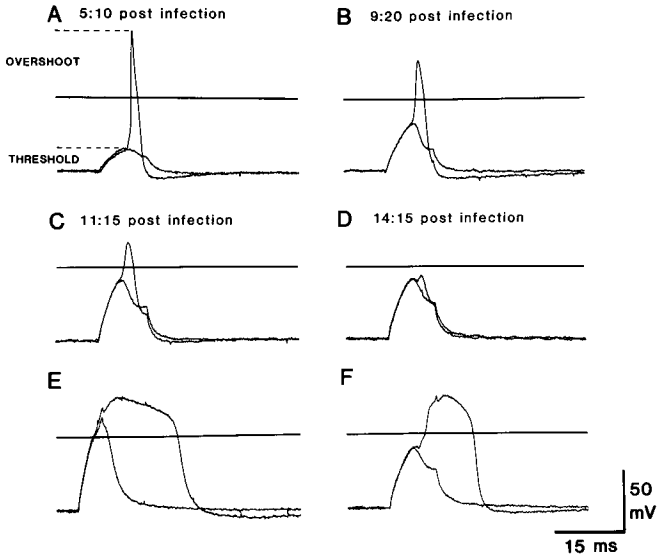

Figure 6. Time course of loss of excitability following infection with the nonsyncytial strain HSV1-C5 syn+. Each panel shows two superimposed traces, on threshold, of action potentials triggered by depolarizing electrotonic potentials 10 msec in duration. The *solid bar* in the center of each panel shows 0 mV transmembrane potential, and individual records are aligned with respect to resting potential. *A–D*, Recorded from four sensory neurons at the times indicated following infection with virus. At 5 hr and 10 min postinfection (*A*), the action potential overshoot and threshold are within normal limits. Traces *B–D* show a progressive increase in threshold and fall in overshoot with time following infection. *E* and *F* show examples of long-duration action potentials triggered by large depolarizations. Traces *D* and *F* were recorded from the same neuron and show residual sodium spikes occurring alone (*D*) or triggering a long-duration action potential (*F*).

To study the onset of these effects, experiments were performed using sets of plates from single-culture preparation, matched for neuronal density and neurite outgrowth. At the start of the experiments, six plates were infected simultaneously as described in Methods; subsequently, individual plates were removed from the incubator at 2.5, 5, 7.5, 10, 12.5, 15 and 24 hr postinfection. Each plate was studied for 2.5 hr, during which period electrophysiological data were recorded from five neurons, and at the end of each recording session plates were fixed with ice-cold methanol for subsequent detection of HSV by immunofluorescence. This procedure was adopted in preference to the alternative of recording from a single plate over a 24 hr plate in order to allow viral replication to proceed undisturbed in cells bathed in growth medium, since the salt solution used for electrophysiological experiments did not contain amino acids and other nutrients required for viral replication.

During the first recording session, 2.5–5 hr postinfection with HSV1-C5 syn+, no changes in action potential threshold or overshoot were detectable (Fig. 6*A*), and inward rectification appeared normal. Subsequently, over the period 5–15 hr postinfection, the threshold for action potential generation progressively increased, and the rate of rise and the overshoot of the action potential decreased (Figs. 6, *A–C*, and 9*A*), until at approximately 16 hr postinfection, neurons became unexcitable in response to current pulses depolarizing the membrane potential by 50 mV. In 10/33 neurons infected with HSV1-C5 syn+, large depolarizing current pulses triggered long-duration calcium spikes with thresholds of approximately 0 mV, i.e., approximately 30 mV depolarized to the threshold for sodium spikes in uninfected neurons. It is possible that this reflects an increase in amplitude of inward calcium currents, since over the membrane potential range −50 to 20 mV, voltage-dependent outward currents were similar in control cells and those infected with HSV1-C5 syn+. This effect on action potential duration was prominent 10–15 hr postinfection, i.e., at a time when low-threshold sodium action potentials are essentially blocked (see Figs. 6 and 9); in some cases, during onset of the virally induced loss of excitability, residual sodium spikes a few millivolts in amplitude were themselves capable of triggering long-duration action potentials (see Fig. 6, *D* and *F*). The time course of onset of the decrease in excitability (i.e., loss of low-threshold action potentials) induced by HSV1-C5 syn+ was essentially identical to the time course of loss of hyperpolarization-activated inward rectification (Fig. 9) and occurred after the intracellular viral

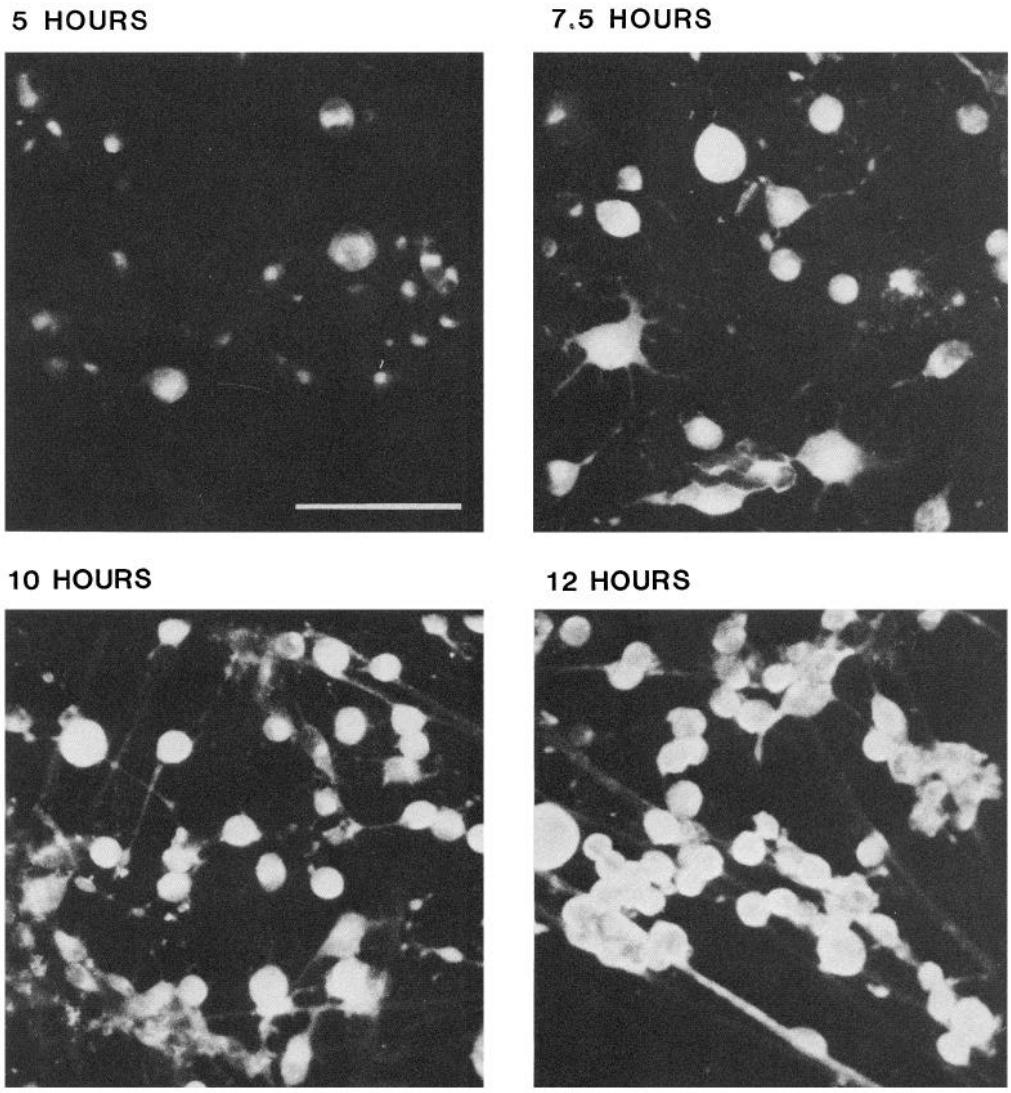

Figure 7. Time course of onset of viral immunofluorescence following infection with the nonsyncytial strain HSV1-C5 syn+. The photomicrographs were obtained from sister cultures used for an electrophysiological study of the onset of loss of excitability induced by HSV1-C5 syn+. At 5 hr postinfection, viral immunofluorescence is detectable in sensory neuron cell bodies and is principally intranuclear. By 10 and 12 hr postinfection, the majority of sensory neuron cell bodies contain viral antigens throughout the cytoplasm. Scale bar, 100 μm.

antigen concentration, as measured with indirect immunofluorescence, appeared to have reached maximal levels (Fig. 6). Thus, virus particles were first detectable 5 hr postinfection with HSV1-C5 syn+, at which time excitability and inward rectification were within normal limits. Intracellular virus antigenicity appeared to reach maximal levels at 7.5 hr postinfection and remained stable over the period in which the loss of excitability subsequently developed.

Replication of the syncytial strain HSV1-03 syn was delayed compared to HSV1-C5 syn+ (compare Figs. 7 and 8), such that sensory neurons were not maximally infected until around 24 hr postinfection, and HSV1-03 syn immunofluorescence was not detectable until 7.5 hr postinfection. The latency to onset of spontaneous activity in cultures infected with HSV1-03 syn was also delayed in time course compared to the loss of excitability produced by the nonsyncytial viruses, and the effect was clearly not maximal at 16 hr postinfection, in contrast to the results obtained with HSV1-C5 syn+ (Fig. 9).

Cultures infected with HSV1-C5 syn+ suffered progressive cell lysis from about 24 hr postinfection (at which time electrophysiological experiments were terminated). In contrast, cultures infected with HSV1-03 syn remained viable for at least 48 hr postinfection, although these cultures were not systematically studied beyond 24 hr.

Viral replication is required for changes in neuronal excitability

The results of the time-course studies suggest that changes in excitability produced by viral infection do not result from either the adsorption or penetration of virus into sensory neurons. The time course of onset of viral replication, as determined by immunofluorescence, appears to precede by several hours the changes in membrane physiology. To confirm that viral replication is a prerequisite for the induction of changes in excitability, we blocked viral DNA synthesis with the specific antimetabolite 9-(2-hydroxyethoxymethyl)guanine (acyclovir), which binds to and competitively inhibits viral thymidine kinase (Elion et al., 1977) after the virus has penetrated the cell membrane and entered the nucleus. Pretreatment of cultures with 50 μM acyclovir for 1 hr prior to infection with HSV1-C5 syn+, coupled with incubation with acyclovir for the rest of the experiment, completely blocked the loss of excitability and loss of

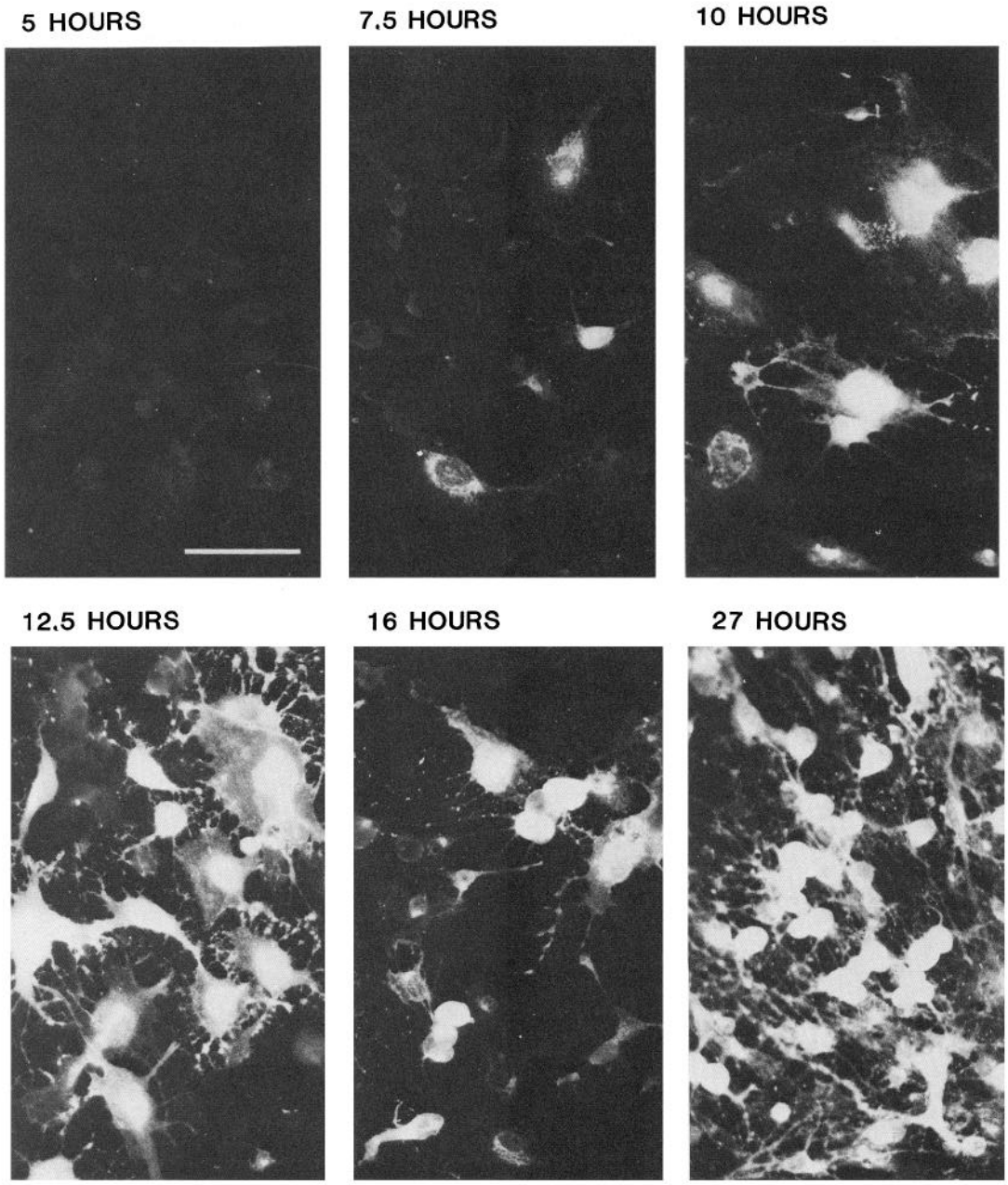

Figure 8. Time course of onset of viral immunofluorescence following infection with the syncytial strain HSV1-03 syn. The photomicrographs were obtained from cultures used for an electrophysiological study of the induction of spontaneous activity by HSV1-03 syn. At 5 hr postinfection, no immunoreactivity was detectable, and all neurons were quiescent. At 7.5 hr postinfection, viral replication is commencing, principally in the nuclei of large nonneuronal cells, and the intensity of this reaction increases at 10 and 12.5 hr. At 16 hr postinfection, viral immunoreactivity was clearly detectable in sensory neurons, the majority of which were labeled by 27 hr. Scale bar, 100 μm.

inward rectification normally produced by this virus (Fig. 10). Pretreatment with acyclovir also prevented the induction of spontaneous activity by HSV1-03 syn (Fig. 11). Control experiments showed that at this concentration 9-(2-hydroxyethoxymethyl)guanine did not affect the excitability of sensory neurons.

Spontaneous activity induced by HSV1-03 syn is synchronous

Sensory neurons *in vivo* do not form synapses within the dorsal root ganglion, and in culture there is no physiological or morphological evidence for synapse formation between sensory neurons (Ransom et al., 1977). Thus, the induction of spontaneous activity and EPSP-like events (see Fig. 4) by HSV1-03 syn is enigmatic in the probable absence of a synaptic mechanism. Since the spontaneous activity is blocked by TTX but persists in low-calcium/high-magnesium solution (Mayer et al., 1985), we have suggested that the cell-fusing properties of HSV1-03 syn cause electrical coupling between sensory neuron axons. Probably as a result of such coupling, a random electrical network forms and supports spontaneous electrical activity because conduction delay in axonal processes provides a mechanism that avoids collision of action potentials, allowing the formation of small reverberating circuits. In control experiments on uninfected sensory neurons, spontaneous action potential activity was occasionally recorded, and this is probably the trigger for activation of the virally induced electrical network. (In contrast to the results presented here, the activity recorded in control experiments was intrinsic to the spontaneously active neurons and showed voltage dependence, being totally blocked by strong

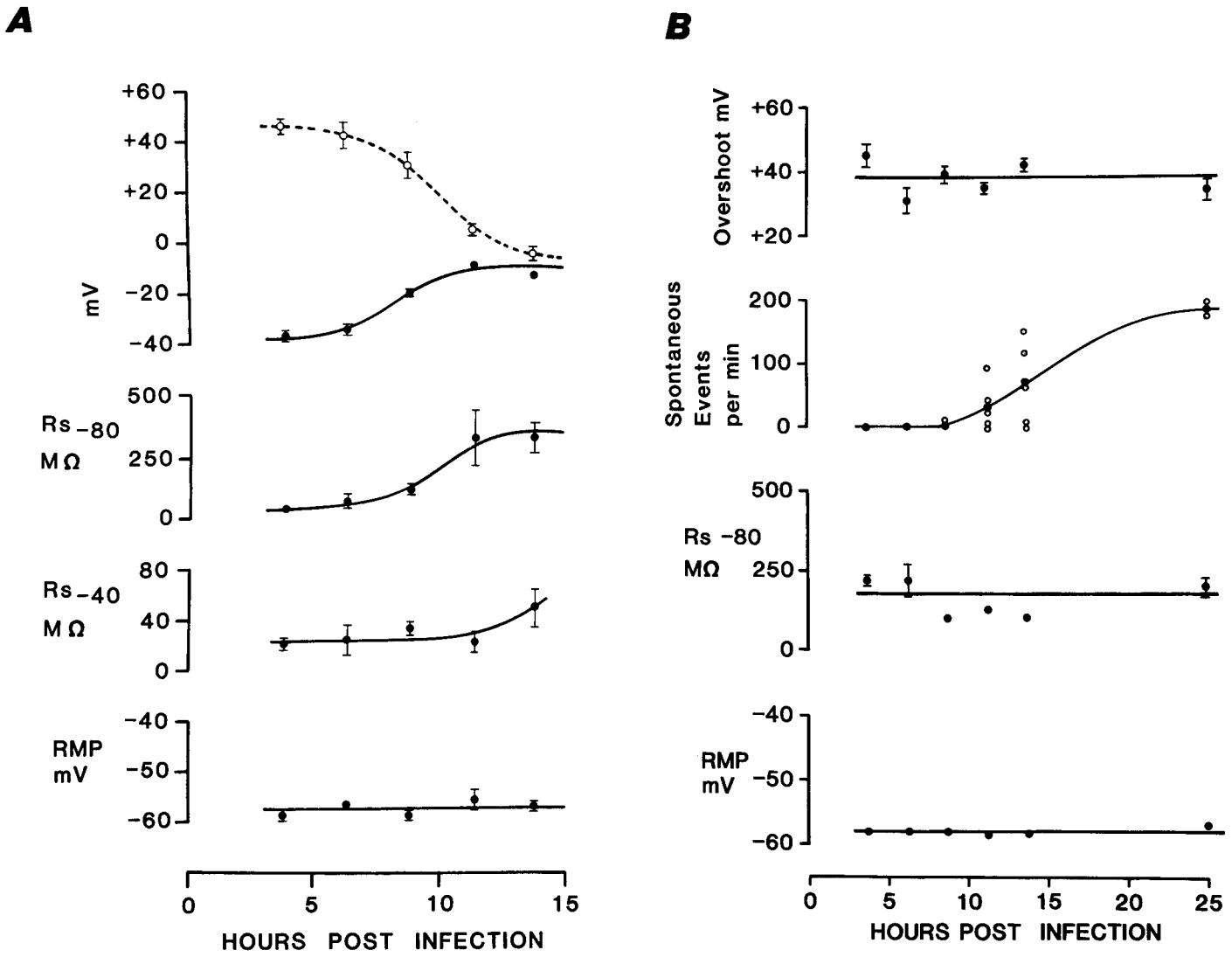

Figure 9. Time course of onset of changes in excitability induced by strains HSV1-C5 syn+ (*A*) and HSV1-03 syn (*B*). *A* (from top to bottom), Action potential overshoot (-----) and threshold (—); slope resistance at −80 mV; slope resistance at −40 mV; and the resting membrane potential. Each data point is the mean of values recorded from five neurons during a 2.5 hr sampling period; *error bars,* SEM. Note the similar time course of the fall in overshoot, increase in threshold, and increase in slope resistance at −80 mV. No change in resting potential occurs over this same time period, while the slope resistance at −40 mV shows only a small increase during the last recording period 12.5–15 hr postinfection. *B* (from top to bottom), Action potential overshoot; frequency of spontaneous activity (measured as the number of action potentials or EPSP-like events recorded during a 5–10 min period); slope resistance at −80 mV; and the resting potential plotted as mean values; *error bars,* SEM. The *open circles* show individual values for the frequency of spontaneous events, which stabilize at around 180/min at 26–27 hr postinfection.

hyperpolarization or replaced by subthreshold membrane potential oscillations with less extreme hyperpolarization.) Further evidence for the electrical network hypothesis is presented below.

In experiments designed to examine interactions between sensory neurons in cultures infected with HSV1-03 syn, marked synchronization of spontaneous activity was recorded in 8/50 pairs, examined as shown in Figure 12. Such synchronous pairs also showed short latency reciprocal excitatory connections in response to action potentials triggered by intracellular current pulses (Fig. 12); synchronous spontaneous activity was also recorded in pairs of neurons in which stimulation-evoked action potentials in one neuron were followed by constant latency action potentials in the second neuron at intervals of 16–145 msec, suggesting that conduction in the network can pass through long axonal pathways, probably via several interneurons. The DC coupling ratio in connected pairs, measured using long hyperpolarizing electrotonic potentials, was usually zero, providing further evidence for remote electrical connections or conduction across a network involving junctions between several neurons.

Discussion

The present results reveal striking strain-dependent differences in the physiological response of sensory neurons to infection with HSV. Two of the viruses, nonsyncytial strains, produced a loss of excitability and a loss of hyperpolarization-activated inward rectification. This effect appears to be specific in that infected neurons maintain a high input resistance, a physiological resting potential, generate broad action potentials (suggesting unimpaired calcium currents), and show depolarization-activated outward rectification. Herpes viruses suppress host cell protein synthesis (Fenwick and Clark, 1982; Sydiskis and Roizman, 1966), and it is possible that membrane proteins with a high turnover would suffer a rapid fall in availability once synthesis was blocked. However, after inhibition of protein syn-

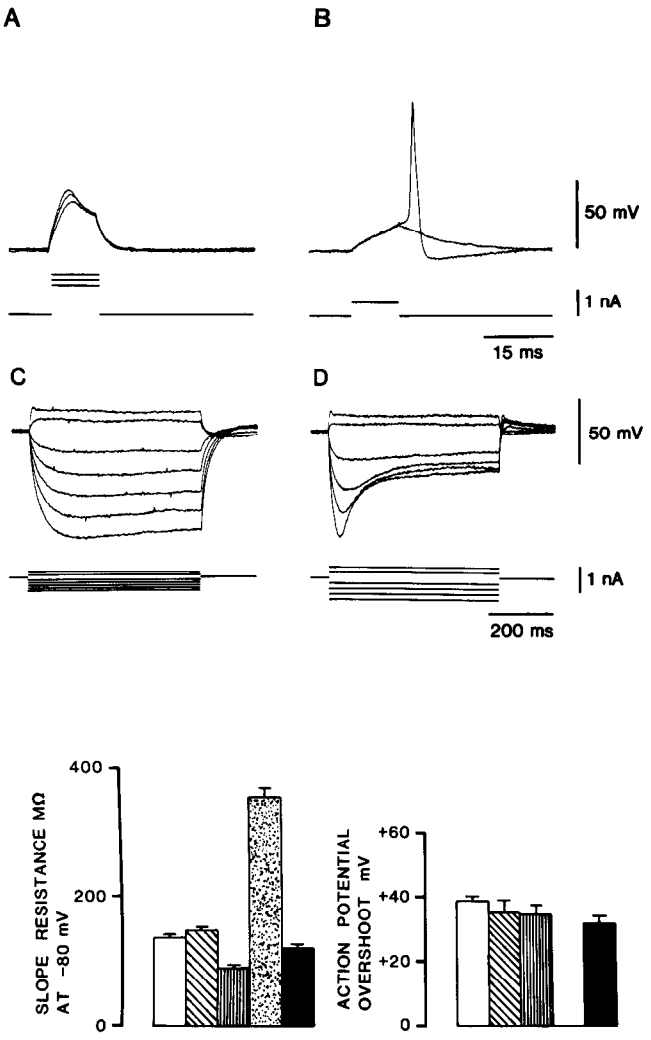

Figure 10. Acyclovir blocks the loss of excitability occurring on infection with HSV1-C5 syn+. *A* and *C*, Traces recorded from neurons in cultures infected with HSV1-C5; *B* and *D*, traces from neurons in cultures infected with HSV1-C5 syn+ in the presence of 50 μM acyclovir to block viral replication. Depolarizing current pulses activate outward rectification in *A*, but in the presence of acyclovir, excitability is maintained and smaller electrotonic potentials trigger normal action potentials (*B*). Hyperpolarizing electrotonic potentials show passive behavior in neurons infected with HSV1-C5 syn+ (*C*), but in the presence of acyclovir, hyperpolarization-activated inward rectification produces large sags in the electrotonic potentials (*D*). The *histograms* shown below summarize the effects of acyclovir on the slope resistance at −80 mV, measured using electrotonic potentials 500 msec in duration (*left*; *control*, □) and the action potential overshoot (*right*) in cultures infected with either HSV1-03 syn (▨, 03 syn; ▥, 03 syn+ ACV) or HSV1-C5 syn+ (▩, C5 syn+; ■, C5 syn+ ACV). These are presented as the mean of values recorded from 9–20 neurons. *Error bars*, 1 SEM. In cultures infected with HSV1-03 syn, acyclovir has no significant effects on excitability or slope resistance at −80 mV.

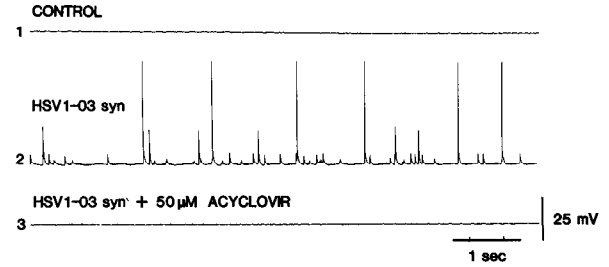

Figure 11. Acyclovir blocks the induction of spontaneous activity by the syncytial strain HSV1-03 syn. Each trace shows a chart record of the membrane potential of a sensory neuron recorded over a 7.5 sec period. The control sample (*trace 1*) and that recorded from a neuron infected with HSV1-03 syn in the presence of 50 μM acyclovir (*trace 3*) do not show any spontaneous activity. In contrast, in the absence of acyclovir (*trace 2*), both subthreshold EPSP-like events and action potentials occur spontaneously.

thesis with cycloheximide, sodium channels in cardiac cell membranes turn over at a rate of less than 5% in a 24 hr period (Renaud et al., 1981), and it seems unlikely that in sensory neurons normal turnover of sodium channels accounts for the complete loss in excitability induced by herpes viruses within 15 hr of infection. It is of interest that the inward rectifier, another conductance mechanism with a high permeability for sodium ions (Mayer and Westbrook, 1983), also inactivates with an identical time course to the loss of the depolarization-activated sodium conductance underlying action potentials. Virus-induced sodium loading of the cytoplasm, and a consequent fall in the driving force for inward sodium current does not seem sufficient to account for the inactivation of sodium-dependent conductance mechanisms: first, there is no change in resting membrane potential during the loss of excitability, suggesting physiological transmembrane ion gradients; second, voltage-clamp recording (Mayer, in press) reveals a genuine loss of hyperpolarization-activated conductance, rather than a change in driving force, to underlie the loss of inward rectification. Further experiments to examine the effect of HSV infection on other sodium-dependent conductance mechanisms—for example, ACh receptors in muscle membranes and excitatory amino acid receptors in neuronal membranes—would be of interest. The present results do not suggest any molecular mechanism for the selective loss of sodium and inward rectifier but not calcium, delayed rectifier, or calcium-activated potassium currents (Mayer, in press) in HSV-infected sensory neurons.

The syncytial strains of virus examined in the present study had quite different effects on membrane excitability from those described above. Strain HSV1-03 syn produced no fall in excitability or loss of inward rectification, despite intense viral antigen synthesis in sensory neuron cell bodies and processes as revealed by immunohistochemistry. In contrast, this strain of virus induced spontaneous activity that was synchronous in pairs of neurons, suggesting widespread interactions between cells that are likely to be electrical in origin (Mayer et al., 1985). However, the fusion reaction underlying such activity is likely to occur at highly localized sites, since the input resistance of neurons in cultures infected with HSV1-03 syn was within normal limits. In contrast, more widespread cell fusion may underlie the loss of input resistance evoked by strain HSV1-17 syn.

General disruption of host cell metabolism seems an unlikely explanation for the virally induced changes in excitability in view of the contrasting effects of the syncytial versus nonsyncytial strains. Experiments on BHK cells infected with the strains of HSV used in this study also reveal strikingly specific effects: At the time of new virus assembly, 10–12 hr postinfection, the glucose transport system is stimulated 2- to 4-fold, intracellular sodium is unaltered, and there is no change in the permeability properties of the plasma membrane (Gray et al., in press; James and Pasternak, in press). Herpes viruses code for approximately 70 proteins, and a possible explanation for the differing response of sensory neurons to infection with strains HSV1-03 syn and HSV1-C5 syn+ would be to suggest that virus-coded proteins expressed selectively in nonsyncytial strains of HSV type 1 may bind to and inactivate sodium and inward rectifier ion channels. Another possibility would be a selective increase in the rate of degradation of sodium and inward rectifier channels following

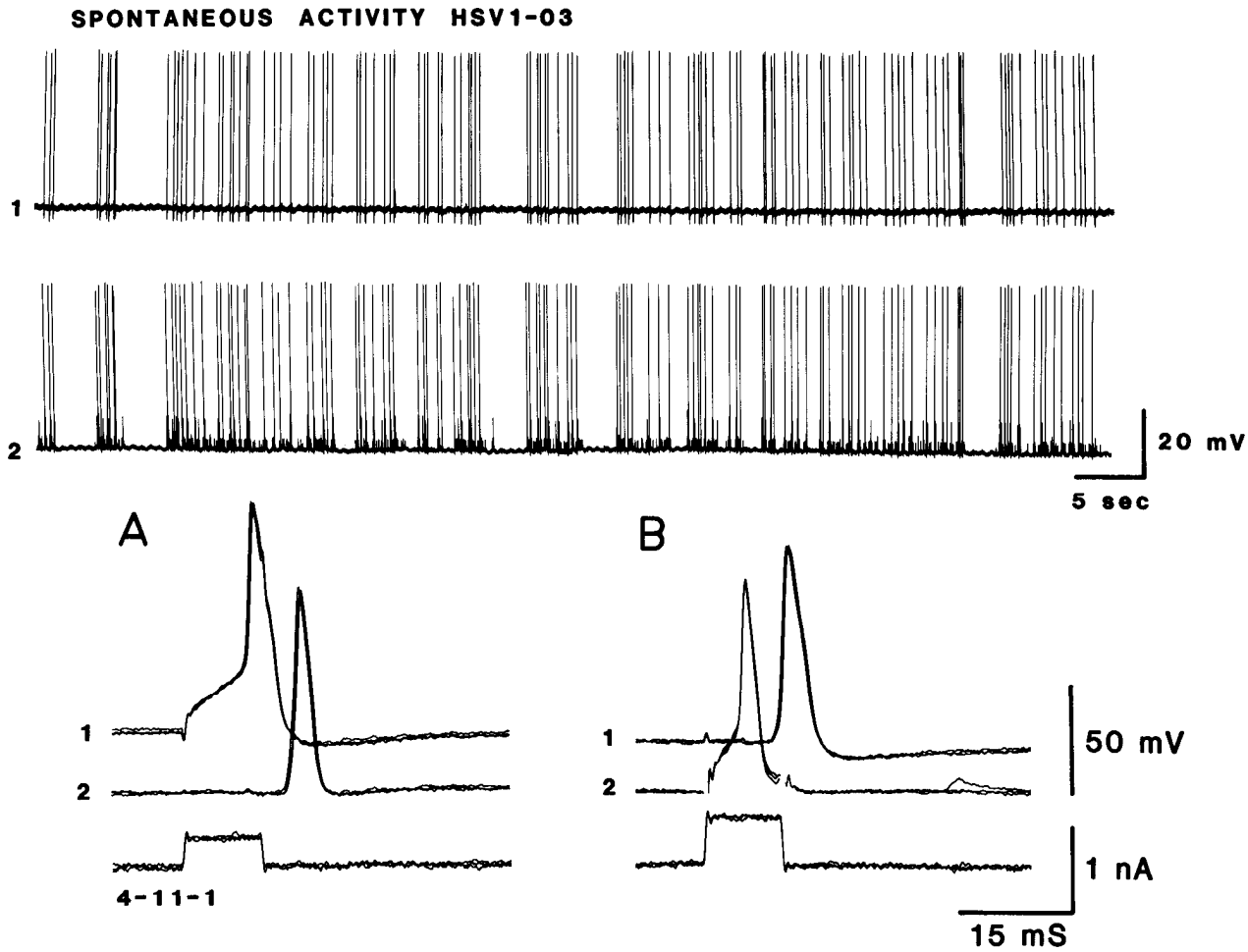

Figure 12. Synchronization of spontaneous activity induced by infection with the syncytial strain HSV1-03 syn. The *upper pair* of chart traces labeled *1* and *2* show simultaneous recordings of the membrane potential of two sensory neurons, both of which are spontaneously active. This activity is highly synchronized. The *lower sets* of oscillographic records show three superimposed traces of action potentials evoked by depolarizing current pulses injected intracellularly. In *A,* the current pulse was injected into neuron *1* (top chart trace in the upper set of records) and evoked an action potential, followed at constant latency by an action potential in neuron *2* (lower chart trace in the upper set of records). In *B,* the intracellular current pulse was injected into neuron *2* and evoked an action potential followed at constant latency by an action potential in neuron *1*; note the spontaneous EPSP-like potential in one of the traces recorded from neuron 2.

infection with the nonsyncytial strains of herpes; a possible mechanism would be via disruption of cytoskeletal elements suggested to anchor ion channels (Almers et al., 1983). Similarly, a possible explanation for the specificity of the response to syncytial strains of the virus would be synthesis of a small number of virally coded glycoproteins triggering membrane fusion. Further characterization of these responses may provide interesting information on both the pathology of virus action and the structure and function of the conductance mechanisms in mammalian neurons.

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