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Loss of Functional Voltage-gated Sodium Channels in Persistent Mumps Virus-infected PC12 Cells

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SUMMARY

Rat pheochromocytoma (PC12) cells, persistently infected with mumps virus (MV), failed to generate full-sized stimulus-evoked action potentials (SEAPs) when examined by intracellular electrophysiological recording techniques. Application of tetrodotoxin (TTX) had little or no effect on MV-reduced SEAPs, indicating that the number of functional voltage-gated Na⁺ channels was decreased or their operation was blocked by the virus. In contrast, MV-infected cells generated normal Ca²⁺ spikes when bathed in a solution containing TTX, tetraethylammonium ions and a high concentration (20 mM) of Ca²⁺. In addition, when infected cells bathed in TTX were superfused with Co²⁺ the SEAP profile reverted to that typical of PC12 cells with functional voltage-gated K⁺ channels only. These observations indicate that MV affects voltage-gated Na⁺ channels, but spares voltage-gated Ca²⁺ and K⁺ channels of persistently infected cells.

Among the RNA viruses that are known to persist in the human central nervous system (CNS), members of the paramyxovirus family are most prominent. Both the measles virus (Haase *et al.*, 1981; Sever, 1983; ter Meulen & Carter, 1984) and the mumps virus (MV) (Valeri *et al.*, 1982) have been implicated in chronic neurological disease associated with persistent infections of the CNS. This disease could be associated with one or more of the several effects which persistent RNA virus infections have on the CNS (Kristensson & Norrby, 1986). Measles virus has been shown to alter membrane receptor-associated neural functions in persistently infected neural cell lines (Halbach & Koschel, 1979).

To determine whether MV could also affect membrane-related functions in persistently infected cell lines, we developed persistent infections of human medulloblastoma (TE671) and rat pheochromocytoma (PC12) cells (Ziegler & Stauffer, 1987). MV-infected (MVI) cultures were found to have a greater proportion of cells that generated stimulus-evoked action potentials (SEAPs) which were smaller in magnitude, had slower depolarization rise times, and were longer in duration than those recorded from mock-infected (MI) control cultures (Ziegler & Stauffer, 1987). These findings were consistent with an altered phase of depolarization and, as such, it was hypothesized that MV might be interfering with the voltage-gated Na⁺ channels which are known to contribute to SEAP electrogenesis in the two cell lines (Dichter *et al.*, 1977; O'Laigue & Huttner, 1980; Syapin *et al.*, 1982). The present study was designed, therefore, to examine this hypothesis by determining which electrogenic channels of PC12 cells might be altered by the MV infection.

Cultures of MI and persistent MVI PC12 cells were prepared and maintained in tissue culture flasks as previously described (Ziegler & Stauffer, 1987). Electrophysiological studies were performed on cultures containing 10⁵ MI or MVI cells, which had been added to collagen-coated 35 mm tissue culture plates and grown in Dulbecco's modified Eagle's medium with 10% foetal

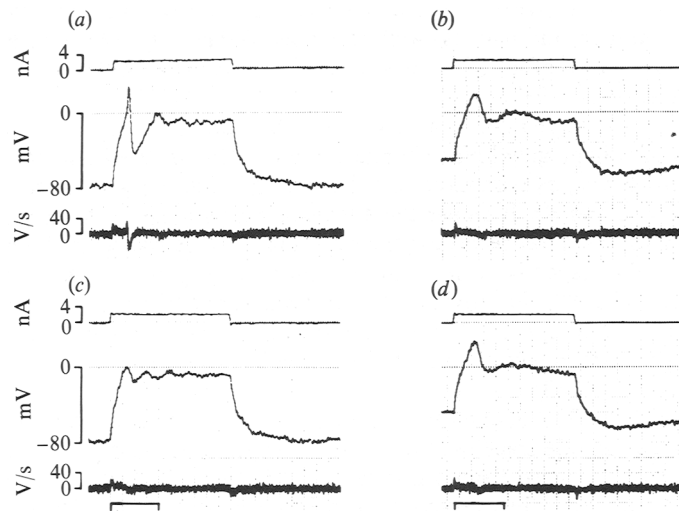


Fig. 1. Effects of TTX on SEAPs from an MI (*a, c*) and an MVI (*b, d*) PC12 cell. Cells were bathed in 20 mM-Ca²⁺ and TTX was applied to each individual cell by micro-pressure-ejection. (*a, b*) 'Control' SEAPs before TTX; (*c, d*) SEAPs after TTX. Top traces, stimulus currents (nA); middle traces, intracellularly recorded membrane voltages (mV); bottom traces, rate of change of membrane voltages (V/s). Scales for all traces are shown on the left. The time scale bars shown at the bottom represent 20 ms.

bovine serum, 5% heat-inactivated horse serum, 20 mg/ml of gentamicin, and 20 µg/ml of mouse 7s nerve growth factor (NGF) for 17 days.

Intracellular recordings of membrane potentials were obtained with fine-tipped glass micropipettes (4 M-KCl, 30 to 50 MΩ) connected to an amplifier with a bridge circuit for injecting stimulating currents. Signals were monitored on an oscilloscope and simultaneously recorded on an FM tape recorder for later analysis. Cells were bathed in a recording solution containing 131 mM-NaCl, 5.9 mM-KCl, 0.8 mM-MgCl₂·6H₂O, 20 mM-CaCl₂, 0.1% glucose and 25 mM-HEPES (pH 7.4, 36 to 37°C). The relatively high Ca²⁺ concentration accentuates Ca²⁺ currents (Moolenaar & Spector, 1979). Resting membrane potentials ranged from -31 to -57 mV. In order to facilitate activation of action potential electrogenic mechanisms, cells were held at -80 to -100 mV prior to stimulation (Dichter *et al.*, 1977; Ziegler & Stauffer, 1987). We also found that PC12 cells would sometimes respond to relatively brief (1 to 5 ms) stimulus pulses. However, the strength of these pulses required to achieve firing threshold was often so large that the rapidly rising upstroke of the SEAP was obscured by the falling phase of the stimulus artefact. Unless noted otherwise, therefore, the more commonly used 50 ms pulse was employed (Dichter *et al.*, 1977; O'Laque & Huttner, 1980). Under these latter conditions, PC12 SEAPs erupt out of the electrotonic response (e.g. Fig. 1*a*) and the entire spike profile is available for analysis. Recording time for each culture was limited to 30 to 45 min. Indirect immunofluorescence staining of methanol-fixed MVI cell cultures with the NP-3 monoclonal antibody to the MV nucleocapsid (Wolinsky *et al.*, 1985) indicated that 90 to 95% of the cells were infected. The data reported herein were obtained from 30 MI and from 35 MVI cells in 10 cultures in three separate experiments.

A typical response of a MI PC12 cell is shown in Fig. 1(*a*). These normal SEAPs were well developed and had relatively (i) large amplitudes, (ii) rapid rates of rise, (iii) brief durations, (iv) deep after-hyperpolarizations (AHPs) and (v) small subthreshold oscillations of the membrane potential superimposed on the electrotonic profile which followed the SEAP. This SEAP profile is identical to those seen in previous studies of normal PC12 cells (Dichter *et al.*, 1977; O'Laque & Huttner, 1980; Rudy *et al.*, 1982; Ziegler & Stauffer, 1987). The responsiveness illustrated in Fig. 1(*a*) was typical of that recorded from all MI cultures prior to any test procedure and/or drug application.

In contrast to the MI cells, SEAPs recorded from MVI cells were smaller in magnitude, slower to rise, longer in duration and had less well developed AHPs (Fig. 1*b*). This abnormal type of responsiveness was typical of all MVI cells tested and was very similar to those of our earlier study (Ziegler & Stauffer, 1987).

If MV affects the voltage-gated Na⁺ channels, then application of tetrodotoxin [TTX, a specific inhibitor of voltage-gated Na⁺ channels (Hille, 1984)] to MVI cells should have little or no effect on their SEAPs, as compared to SEAPs of MI cells. A typical result of this test is illustrated in Fig. 1(*c* and *d*). It shows the effects of superfusing TTX on the same two cells shown in Fig. 1(*a* and *b*). After recording their responses, TTX was 'puffed' (20.7 kPa, 1 to 3 s) over the surface of the respective cells using micro-pressure-ejection techniques (McCaman *et al.*, 1977; Palmer *et al.*, 1980). The toxin (10⁻⁵ g/ml) was applied from a second micropipette (tip diameter 3 μm) positioned approx. 35 mm from the cell. These ejection parameters eliminated any hydrostatic pressure-induced artefacts in the voltage profile (Choi & Fischbach, 1981; Smith & Cunningham, 1983).

Fig. 1(*c*) shows that TTX blocked the rapidly rising Na⁺ spike of the normal SEAP. The intracellular voltage (middle trace) lost the positive-going overshoot potential and the rate of change of voltage was reduced from a normal value of approx. 40 V/s to a barely noticeable inflection (lower trace). Both the overshoot potential (Hodgkin & Katz, 1949) and the upstroke velocity (Hodgkin & Huxley, 1952) of SEAPs are considered to be direct functions of active Na⁺ channels. Their absence and reduction, respectively, reflect a loss and reduction of Na⁺ currents. The residual waveform, however, was qualitatively similar to that of an untreated 'control' MVI cell, i.e. only the relatively small and slow subthreshold voltage oscillations remained superimposed on the electrotonic responses (e.g. Fig. 1*b*). It is particularly significant, however, to note that TTX did not appreciably change the abnormal waveform of an MVI cell SEAP; the responses before and after treatment with TTX are nearly identical (Fig. 1*b* and *d* respectively). In one experiment, this result (i.e. no effect of TTX) was seen in six of six MVI cells. In another experiment, two cells showed no effect while in eight there was a very slight decrease of the already virally reduced, initial phase of the SEAP (not shown). Thus, in some infected cells, a small unknown fraction of Na⁺ channels were unaffected by the virus and remained functional. The mechanism by which the virus apparently spared some Na⁺ channels is unknown. One possibility may be that the infection did not produce viral proteins in sufficient quantities to compromise all of the available Na⁺ channels (Fukuda *et al.*, 1983*a,b*).

The excitable membranes of PC12 cells also contain voltage-gated Ca²⁺ and voltage-gated K⁺ channels (O'Lague & Huttner, 1980; Arner & Stallcup, 1981). We investigated, therefore, the possibility that MV might also affect these channels. To examine the voltage-gated Ca²⁺ channels, the Na⁺ and K⁺ channels of MI and MVI cells were first blocked by including TTX (10⁻⁵ g/ml) and 20 mM-tetraethylammonium ion (TEA) [a blocker of voltage-gated K⁺ channels (Hille, 1984)] in the recording solution which bathed the cells. Fig. 2(*a*) and (*b*) show the responses recorded from MI (n = 13) and MVI (n = 14) cells respectively, under these conditions. For these particular studies SEAPs could be evoked in response to stimulus pulses of relatively short duration (1 to 5 ms) and because of the slower rising upstroke of the Ca²⁺ spike, the entire waveform could be distinguished from the stimulus artefact and SEAPs were evoked from the resting state because the cells stopped firing (i.e. the Ca²⁺ spikes disappeared) when they were held at a hyperpolarized level. These relatively slow (10 to 30 V/s) and prolonged (50 to 60 ms) responses were typical of Ca²⁺ spikes seen in PC12 cells (O'Lague & Huttner, 1980). In addition, micro-pressure-ejection (1 to 2 s) of 10 mM-Co²⁺ [a reversible blocking agent of Ca²⁺ channels (Hille, 1984)] completely eliminated these responses for 30 to 90 s, after which time they began to reappear as Co²⁺ diffused away from the ejection site (not shown). More importantly, however, a comparison of the traces shown in (*a*) and (*b*) in Fig. 2 shows that there was virtually no difference between the MI and MVI cells. Since there was no change in the responsiveness to Ca²⁺ these data indicate that MV did not affect the Ca²⁺ channels.

A similar approach was used to examine the effects of virus on voltage-gated K⁺ channels. MI and MVI cells were bathed in the recording solution containing 10⁻⁵ g/ml of TTX to block the Na⁺ spike. As expected, the responses of the cells (Fig. 3*a, b*) were quite similar to those

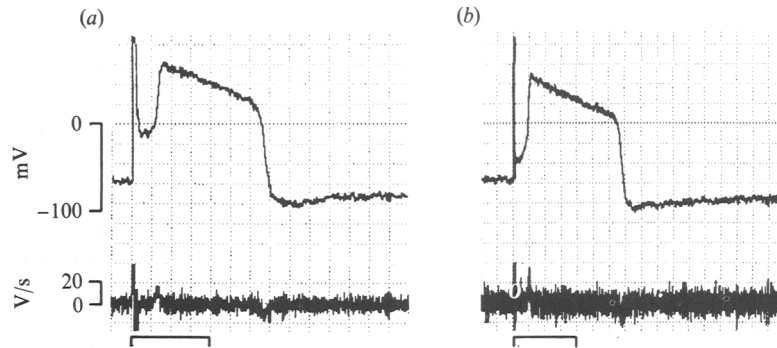


Fig. 2. Effect of MV on Ca^{2+} spikes of PC12 cells. Intracellular responses recorded from MI (a) and MVI (b) PC12 cells bathed in TTX, TEA and high concentration (20 mM) Ca^{2+} . Upper traces, membrane voltage (mV); lower traces, rate of change of membrane voltages (V/s). The time scale bars represent 40 ms; note that it is slightly expanded in (a).

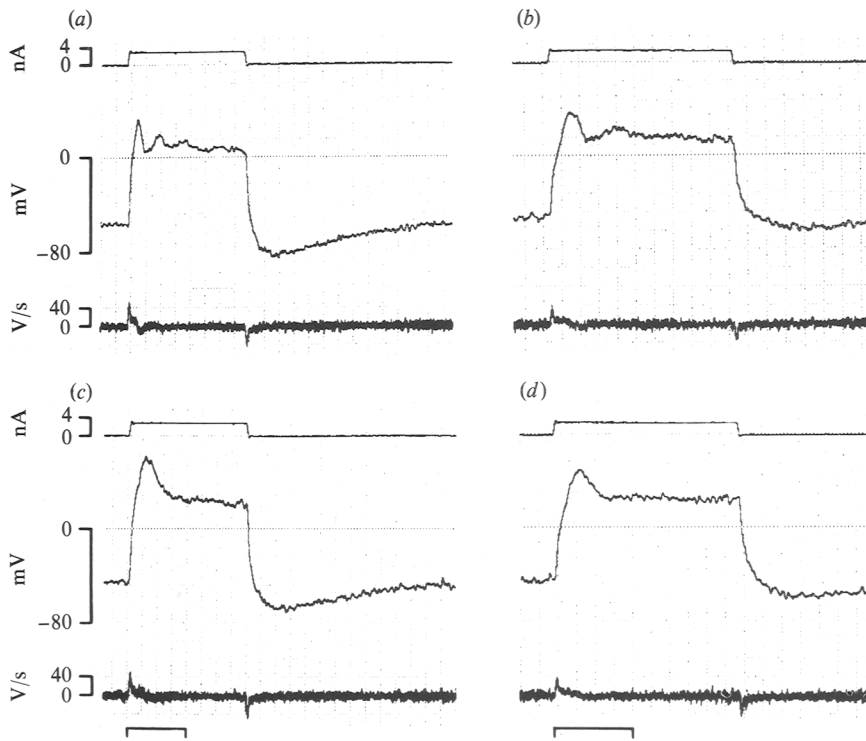


Fig. 3. Effect of MV on voltage-gated K^{+} channels of PC12 cells. Micro-pressure-ejection of Co^{2+} on MI (a, c) and MVI (b, d) PC12 cells. Cells were bathed in control solution containing TTX and 20 mM- Ca^{2+} . (a, b) Control responses; (c, d) test responses (10 mM- Co^{2+}). Top traces, stimulus currents (nA); middle traces, intracellular voltages (mV); bottom traces, rate of change of membrane voltages (V/s). Scales for all traces are shown on the left. Time scale bars shown at the bottom represent 20 ms. The scale in (d) is slightly expanded in time.

observed when TTX was micro-pressure-ejected on the cells, i.e. only small (20 to 30 mV), slowly oscillating (5 to 15 V/s), subthreshold changes of the membrane potential were present (e.g. Fig. 1c, d). When the voltage-gated Ca^{2+} channels were momentarily blocked by micro-pressure-superfusion of 10 mM- Co^{2+} the 'control' responses shown in Fig. 3(a, b) were converted into those of the test, shown in Fig. 3(c, d). These latter waveforms are very similar to those recorded

from PC12 cells, in which voltage-gated K^+ channels are the dominant ionic conductors (O'Lague & Huttner, 1980; Rudy *et al.*, 1982). This is evident by the fact that the membrane potential has a temporal profile which is considered to be typical of delayed rectification, i.e. an initial electrotonic peak which is terminated by the action of the voltage-gated K^+ conductance (Rudy *et al.*, 1982) and that there is a relatively long lasting AHP at the end of the response (O'Lague & Huttner, 1980). As before with the Ca^{2+} spike, there was no difference between the MI and the MVI cells (Fig. 3c and d respectively). These observations indicate, therefore, that the voltage-gated K^+ channels have not been altered significantly by MV.

Our data show that MV affects the voltage-gated Na^+ channels, but apparently spares the voltage-gated Ca^{2+} and K^+ channels. These effects of MV in PC12 cells are similar to those reported for herpes simplex virus (HSV) in dorsal root ganglia cells in which HSV also specifically reduced or eliminated the Na^+ spike, but did not alter the Ca^{2+} potentials (Fukuda *et al.*, 1983b; Oakes *et al.*, 1981). We have also seen similar effects of MV on the Na^+ channel in TE671 cells (Stauffer & Ziegler, 1988). It seems, therefore, that the viral effect is a specific rather than a general one with the Na^+ channel being particularly susceptible. [To date, the available data do not exclude an effect on the Ca^{2+} -activated K^+ channels which are also considered to be present in PC12 cells (O'Lague & Huttner, 1980; Arner & Stallcup, 1981).] Although several different types of Na^+ channels have been identified pharmacologically (Strichartz *et al.*, 1987), it may be that some particular site on the channels' functional structure is relatively common to most Na^+ channels and is unusually susceptible to virus-induced alterations.

An interesting question is why the virus should affect only the Na^+ channel. Since electrogenesis is a membrane phenomenon, we believe that the MV envelope proteins are responsible. Current concepts of enveloped virus replication hold that these proteins are inserted rather randomly into specific membranes of the host cell; however, maturation can occur at restricted membrane domains in polarized cells (Compans, 1984). It would seem, therefore, that MV envelope proteins would exert their effect equally on all channels. Instead, it appears that a viral protein specifically disables the Na^+ channel. Perhaps the individual proteins which form the channel have a propensity for interacting directly with viral envelope proteins, or perhaps the channel gating mechanisms are susceptible to viral protein in close proximity.

Functional Na^+ channels are only expressed in PC12 cells after NGF treatment (Dichter *et al.*, 1977; Rudy *et al.*, 1982) so that channel synthesis or maturation could also be affected by persistent infections. Acute MV infection, however, alters SEAP waveforms in a manner similar to that observed with the persistent infections (Ziegler & Stauffer, 1987). This observation suggests that pre-existing channels are the viral substrate, not the biosynthetic process; however, the latter hypothesis cannot yet be excluded. Several additional possibilities have been proposed for mechanisms by which the Na^+ channel might be affected by viral infections (Fukuda *et al.*, 1983b).

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