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Reduction of voltage-dependent calcium currents in mumps virus-infected cultures of rat hippocampal neurons

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The effects of a mumps virus infection on functional properties of embryonic hippocampal neurons in culture were analysed with special emphasis on voltage-dependent Ca^{2+} channels. Cultures with higher or lower density of glial cells (not treated or treated with mitotic inhibitor, respectively) were infected with the relatively non-cytolytic RW strain of mumps virus and currents were recorded from neurons using whole cell voltage clamp. More than 65% of neurons and glial cells contained viral antigens 1–2 days post infection (p.i.). Glial cells remained infected 6–7 days p.i., while the ratio of infected versus uninfected neurons, especially in cultures with higher glial cell density, was reduced. In both infected and uninfected cultures the somal voltage-dependent Ca^{2+} currents were stronger in cultures with a higher glial cell density, which indicates that these currents are influenced by glial cells. Introduction of the virus into cultures caused a selective decrease in inward Ca^{2+} currents, which was most marked at days 6–7 p.i., and which included both infected and unifected neurons. Spontaneous synaptic currents and other ion channel conductances appeared normal in the infected cultures. Dantrolene, which inhibits release of Ca^{2+} from intracellular stores, decreased the neurons that died during the infection. Taken together the results show that a mumps viral infection can selectively alter the number or function of somal voltage dependent Ca^{2+} channels in immature hippocampal neurons and that this may reflect a disturbed glia-nerve cell interaction.

Keywords: nervous system; glial cells; voltage clamp; viral infection

Introduction

Paramyxoviridae, such as measles and mumps virus, have the potential to infect the nervous system both in humans and in experimental animals (Wolinsky *et al*, 1976). Measles virus infections, particularly when they occur during early life, may not rapidly be cleared but persist for various periods of time in the brain. Occasionally, individuals harbouring such infections may later in life develop progressive brain diseases such as 'subacute measles encephalitis' which occurs in young immunocompromised patients (reviewed by Hughes *et al*, 1993; Mustafa *et al*, 1993), and subacute sclerosing panencephalitis. Also following mumps virus infections, slow progressive infections of the brain have been reported and neurological sequelae after mumps encephalitis are not rare (Vaheri *et al*, 1982; Julkunen *et al*, 1985; Haginoya *et al*, 1995; Nussinovitch *et al*, 1995). The factors that trigger the onset of the progressive diseases and the mechanisms that lead to neuronal dysfunctions and degeneration in these as well as in other persistent virus infections remains to be clarified. Thus, it is of interest to find out if disturbances in differentiated neuronal functions can occur after infection with paramyxoviruses.

Ca^{2+} channels play a pivotal role in the normal function of the neuron as well as during neuronal differentiation and degeneration. Voltage-dependent Ca^{2+} channels take active part in regulating the shape and frequency of the action potential, and in the triggering of presynaptic release of neurotransmitters (Hille, 1992), while ligand-gated Ca^{2+}-permeable channels, such as *N*-methyl-D-aspartate (NMDA) receptors, are involved in synaptic transmission. Ca^{2+} is also an important intracellular messenger which regulates a number of processes including the activation of Ca^{2+}-dependent enzymes (Miller and Kennedy, 1986), the mobilisation of Ca^{2+} from intracellular stores (McPherson *et al*, 1991)

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and gene expression by signalling to the nucleus (Bading *et al*, 1993). In recent years a wealth of data have accumulated on disturbances in Ca^{2+} homeostasis in neurons following exposure to human immunodeficiency virus HIV-1 antigen, i.e. gp 120, gp 160 and Tat, and it has also been shown that treatment with NMDA antagonists or blockers of voltage-dependent Ca^{2+} channels may rescue neurons from degeneration caused by the HIV-1 antigens (Dreyer *et al*, 1990; Lipton, 1994; Lannuzel *et al*, 1995; Nath *et al*, 1996).

In a previous study on Ca^{2+} currents during mumps virus infections in embryonic dorsal root ganglia (DRG) neurons, a reduction in Ca^{2+} influx during the action potential was found in the early phase of infection (Maehlen *et al*, 1991). It has also been shown for these cells that nifedipine, which is a blocker of L-type voltage-dependent Ca^{2+} channels, has neuroprotective effects during infection with mumps virus (Andersson *et al*, 1991). However, different populations of neurons differ in the number, cellular distribution as well as types of Ca^{2+} channels (Hillman *et al*, 1991; Gohil *et al*, 1994). It is therefore important to examine the effect of mumps virus infection also on neurons in the central nervous system which are the targets for the natural infections. In the present study, we chose hippocampal neurons because they are well characterised regarding the content and distribution of Ca^{2+} channel subtypes (Elliott *et al*, 1995) and appear to be particularly sensitive to Ca^{2+} cytotoxicity (Meldrum, 1986). Here we report that mumps virus infections in rat primary cultures of hippocampal neurons cause a reduction of neuronal inward voltage-dependent Ca^{2+} currents and that these changes may be related to disturbances in the glia-nerve cell interactions.

Results

Ca^{2+} currents were identified in the cultured hippocampal neurons by using ion channel blockers in a series of control experiments designed for this purpose. Action potentials, which normally are derived from voltage activated Na^+ and K^+ channels, could be evoked in these cells as shown in Figure 1a. When Na^+ channels were blocked with tetrodotoxin (TTX; 1.5 μM, n=5) the large inward currents seen in response to increasing voltage steps were eliminated as demonstrated by Figure 1b, c. The remaining outward currents which develop during the step (Figure 1c) were greatly reduced or blocked by using Cs^+ filled pipettes.

When both inward voltage activated Na^+ and outward K^+ currents were blocked in this manner, a clear inward current could be seen as a response to voltage steps as shown in Figure 1d (note the difference in scale sensitivity from Figure 1b, c). It was voltage-dependent because it appeared only in response to voltage steps and only at potential steps above a certain value. Further, as step potentials were increased the inward current decreased after reaching a maximum which is indicative of an ion channel permeable to a species with an equilibrium potential near or above 0 mV, such as Na^+ or Ca^{2+}. This current was much reduced or eliminated in 11 examined cells by adding to the bath either Co^{2+} (2 mM) or Cd^{2+} (0.5 mM), both of which are known Ca^{2+} channel blockers (Figure 1e). In addition, TTX and the use of Cs^+-filled pipettes elicited depolarizing plateau potentials like that shown in Figure 1f by appropriate stimuli during current clamp recordings, but not with either of the two Ca^{2+} channel blockers present.

The inward, voltage-dependent Ca^{2+} currents seen in the presence of TTX and with Cs^+-filled pipettes were measured in control cultures and in those infected with mumps virus. The cells were stimulated with a series of 8 voltage steps (20 ms) from -30 to +5 mV in 5 mV increments using a holding potential of -60 mV. The method of measurement is illustrated in Figure 2 which shows seven current responses of a control cell and for a cell from an infected culture at day 6 p.i. (both not exposed to mitotic inhibitor). It is clearly seen that during the voltage steps the infected cell had less maximum inward current than the control cell.

Figure 3 is a summary of the measurements illustrated in Figure 2 made from control and infected cultures at either 1–2 or 6–7 days p.i. To examine the influence of glial cells on Ca^{2+} currents, some of the cultures were treated with the mitotic inhibitor 5-fluorodeoxyuridine, which reduced the density of glial cells (cf O'Malley *et al*, 1994). In cultures treated with the mitotic inhibitor, the glial cell layer was thin and did not reach complete confluence, whereas in cultures not treated with the mitotic inhibitor the glial cell layer was thick and confluentic. The majority of glial cells consisted of astrocytes as determined by immunohistochemistry. In control cells the inward, voltage-dependent Ca^{2+} currents were significantly stronger ($P<0.001$, t-test) in cultures not treated with mitotic inhibitor (811 ± 66 pA; n=26; Figure 3) compared to cultures treated with mitotic inhibitor (522 ± 37 pA; n=68; Figure 3). In each of the two control groups, there was no statistical difference in Ca^{2+} currents when comparing days 1–2 or 6–7 (P=0.28, t-test).

After infection, cultures not exposed to mitotic inhibitor exhibited already at day 1–2 p.i. a significant decrease in Ca^{2+} currents (480 ± 57 pA; n=30; Figure 3; $P<0.001$, one-way analysis of variance (ANOVA), followed by Dunnett's t-test) compared to control cells which persisted at days 6–7 p.i. (433 ± 35 pA; n=72; Figure 3). In infected cultures exposed to mitotic inhibitor, there was a slight, but not significant, decrease in Ca^{2+} currents at days 1–2 p.i. (421 ± 43 pA; n=51; Figure 3). At days 6–7 p.i. there was, however, a significant

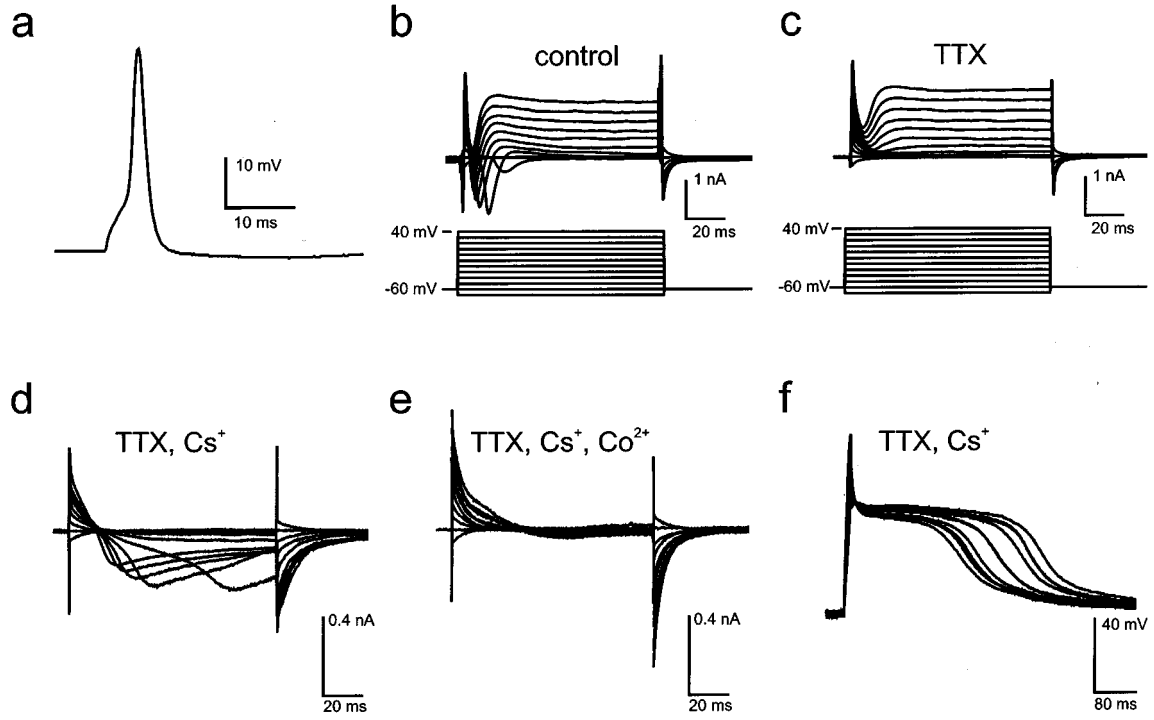

Figure 1 Isolation of ion currents. (**a**) An action potential recorded from a control hippocampal cell during whole cell patch under current clamp in response to a short pulse of stimulus current. Resting potential was −58 mV. Normal intra- and extracellular physiological solutions were used in the bath and pipette respectively. (**b**) Current records (upper traces) in response to voltage steps (lower traces) from a holding voltage of −60 mV in the same cell as in (**a**) during voltage clamp. Clear rapid inward (downward) current peaks are followed by delayed outward currents during the steps. (**c**) Voltage clamp records from the same cell as in (**a**) and (**b**) after blocking Na^+ channels with TTX (1.5 μM) added to the bath. Voltage clamp parameters were the same as in (**b**). The inward current peaks are no longer present but the outward delayed currents remain. (**d**) Currents recorded from a different cell in response to voltage steps from −70 to 10 mV from −60 mV holding potential where, in addition to TTX application, K^+ is replaced by Cs^+ in the patch pipette. Outward currents are much reduced compared to (**b**) and (**c**) and an inward current peak can be seen to appear with the smallest then largest amplitude which gradually diminishes as the voltage steps are closer to 10 mV. Note the difference in vertical calibration scale from (**b**) and (**c**). (**e**) Current records obtained from the same cell as in (**d**) after Co^{2+} (2 mM) has been added to the bath to block Ca^{2+} channels. All other conditions were the same as in (**d**). The inward currents have been largely reduced. (**f**) Voltage responses to a current stimulus during current clamp obtained from a neuron in TTX (1.5 μM) with a Cs^+ filled pipette. A large depolarizing plateau follows a much shorter stimulus pulse represented by the sharp positive going peak. The cell's potential was maintained at −60 mV between responses. Leak current was subtracted from voltage clamp records in (**b**, **c**, **d** and **e**).

decrease (233 ± 33 pA; n=31; Figure 3; $P<0.001$, one-way ANOVA, followed by Dunnett's t-test) in Ca^{2+} currents compared to control cells. There was no statistical difference in Ca^{2+} currents between neurons with distinct immunolabelling for mumps virus infection (Figure 4) and those that were not labelled. Thus, in cultures treated with mitotic inhibitor the Ca^{2+} currents were 348 ± 31 pA in cells showing immunolabelling (n=31) and 441 ± 109 pA in cells without labelling (n=20) at days 1–2 p.i. (P=0.42, t-test), while the values at days 6–7 p.i. were 222 ± 31 pA in cells with immunolabelling (n=19) and 249 ± 70 pA in cells without labelling (n=12; P=0.73, t-test). In cultures not treated with mitotic inhibitor the Ca^{2+} currents at days 1–2 p.i. were 519 ± 76 pA in immunopositive cells (n=17) and 427 ± 87 pA in immunonegative cells (n=13; P=0.43, t-test), while at days 6–7 p.i. none of the examined cells (n=72) showed immunopositive labelling.

In cultures exposed to the mitotic inhibitor, 77 ± 3% and 65 ± 3% of the neurons in infected cultures contained viral antigen at days 1 and 6 p.i., respectively, as examined by double-immunolabelling with antibodies against mumps virus and microtubule-associated protein 2 (MAP 2). In cultures not exposed to mitotic inhibitor, hence with a higher density of glial cells, 66 ± 3% and 15 ± 2% of the neurons in infected cultures contained viral antigen at day 1 and 6 p.i., respectively. Most glial cells (>70%) were infected in all cultures exposed to virus (Figure 4).

Another indication that voltage-dependent Ca^{2+} currents were reduced in infected cultures was the inability to produce depolarizing Ca^{2+} plateaus like those shown in Figure 1f. The stimulus pulses for eliciting such responses under current clamp conditions were done in over one third of the cells and included varying the membrane potential

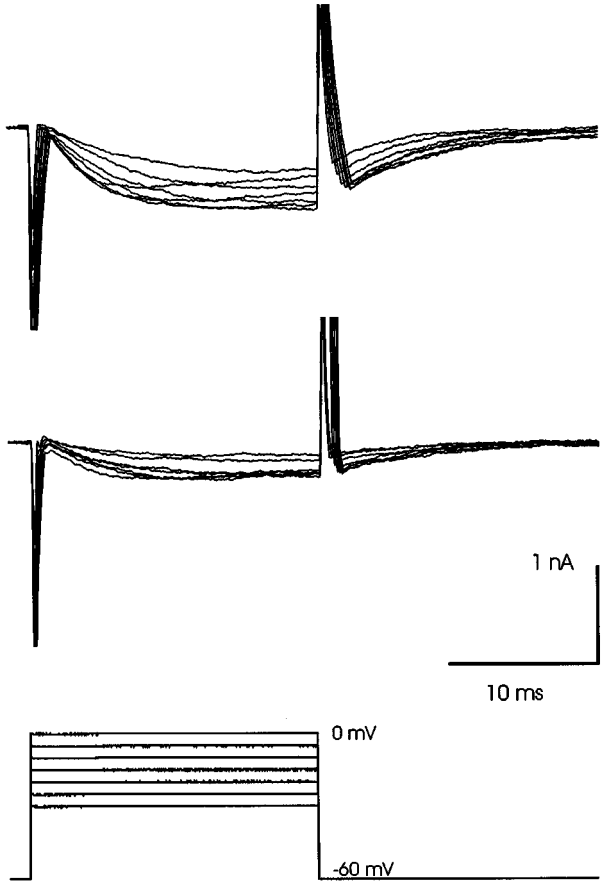

Figure 2 Ca^{2+} current responses in a control cell (top panel) and a cell from an infected culture (middle panel) to voltage steps (bottom panel) in TTX using Cs^{+} filled pipettes. The responses shown are to seven voltage steps with 5 mV increments starting from −30 mV using a holding potential of −60 mV. Leak current has been subtracted. Note the reduced Ca^{2+} currents in the cell from an infected culture.

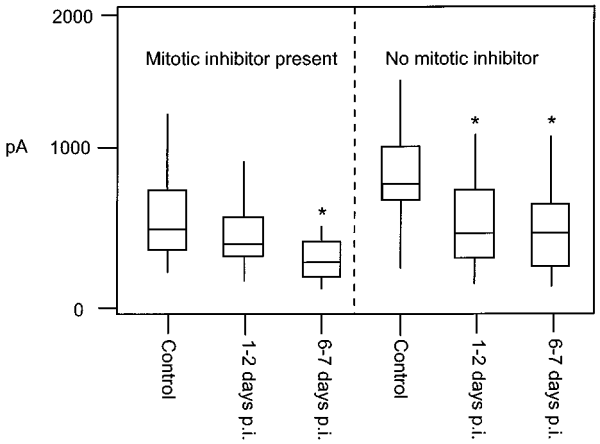

Figure 3 Boxplot showing Ca^{2+} currents in hippocampal neurons in control cultures, and in cultures infected with mumps virus at days 1–2 and 6–7 post infection (p.i.). The cultures are divided in two groups: treated or not treated with the mitotic inhibitor 5-fluorodeoxyuridine. Each boxplot gives the median, the first and third quartile and whiskers. Values were analysed in the two groups with one-way ANOVA followed by Dunnett's *t*-test post hoc analysis; significance indicated by an asterisk. Note the reduction in Ca^{2+} currents in both groups after infection; in the cultures not treated with mitotic inhibitor reduction was already present at days 1–2 p.i. Note also that Ca^{2+} currents were larger in cultures not treated with mitotic inhibitor.

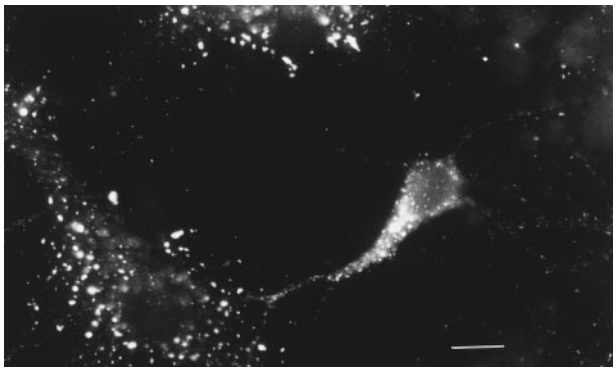

Figure 4 Rat hippocampal neuron at day 1 p.i. immunohistochemically labelled with a rabbit anti-mumps hyperimmune serum followed by a FITC-conjugated antibody in order to show infected cells. Note the appearance of virus throughout the neuritic processes. Two infected glial cells are also visible, one at the upper border (only part of the cell visible) and one at the lower left margin. The cell culture had not been treated with mitotic inhibitor. Scale bar=10 μM.

between −70 mV and −50 mV during current pulses. Tests for the presence of these calcium plateau potentials showed that in infected cultures at day 6–7 p.i. (treated with mitotic inhibitor) they could not be induced whereas, with few exceptions, they were in control cells.

The question of whether the reduced voltage-dependent Ca^{2+} currents in infected cultures was due to a general deterioration of the neurons caused by the virus was addressed from several aspects. During the course of the study the neurons were found to have frequent spontaneous synaptic potentials, often leading to action potentials, as well as synaptic currents under voltage clamp when bathed in normal physiological solutions. In the presence of TTX the action potentials were abolished, but smaller, rapid spontaneous current peaks remained which were mainly inward at holding potentials of around −60 mV. The current reversed direction at holding potentials above 0 mV, which would be expected of an ionic conductance to Na^{+}, Ca^{2+} or both. 2-amino-5-phosphonovalerate (APV; 100 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 50 μM), which block postsynaptic glutamate receptors, decreased the amplitudes of these current responses, indicative of action at postsynaptic glutamate receptors. They are, thus, likely arising from postsynaptic conductance increases caused by spontaneous transmitter release. During

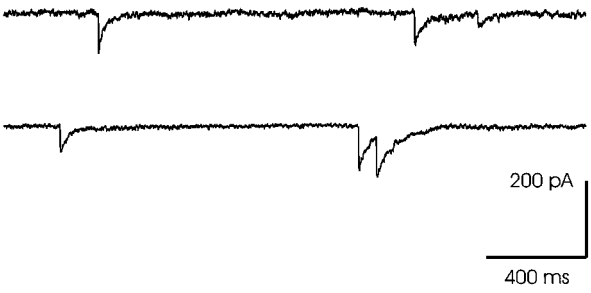

Figure 5 Spontaneous synaptic currents recorded under voltage clamp from hippocampal cells in the presence of TTX (1.5 μM) and using Cs^+ filled electrodes. Synaptic currents recorded from a control cell (upper trace) and from a cell in an infected culture (lower trace). The holding potential was -60 mV. Note the similarity between frequency and shape of single current peaks.

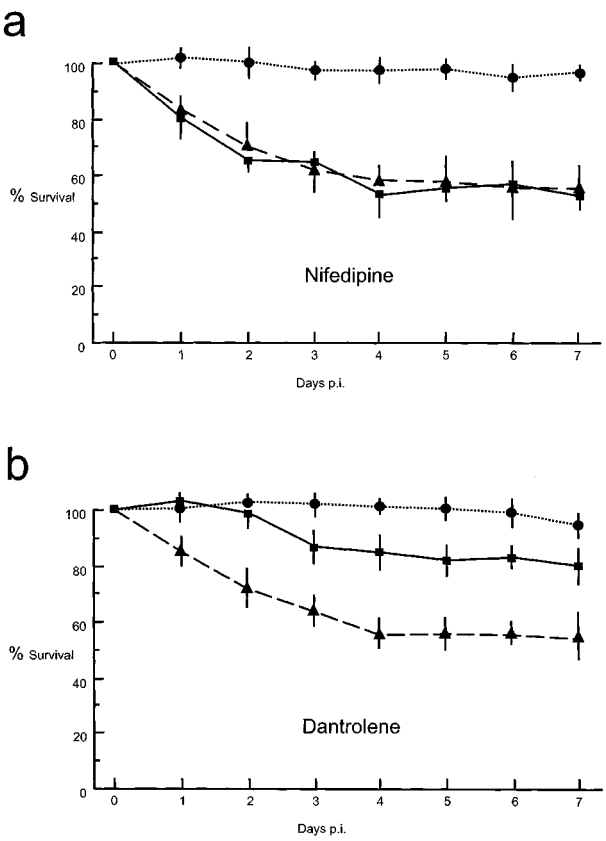

Figure 6 (**a**) Survival of neurons (percentage of the original numbers) in mumps virus-infected cultures treated (solid lines - squares) and not treated (dashed line - triangles) with 50 μM nifedipine. The data represent compiled values from six experiments presented as mean $\pm$ s.e.m. No significant difference is noted between the two groups. Uninfected, nifedipine treated neurons (dotted line - circles) survived. (**b**) Survival of neurons in mumps virus-infected cultures treated (solid line - squares) and not treated (dashed line - triangles) with 30 μM dantrolene. The data are presented as mean $\pm$ s.e.m. and are compiled from six different experiments. The nerve cell loss of the dantrolene treated nuerons was significantly less during the whole observation period (days 1 and 2: $P<0.001$, days 3–7: $P<0.05$, Mann-Whitney U-test). Uninfected, dantrolene treated neurons (dotted line - circles) survived.

the experiments on Ca^{2+} influx in control and infected cultures, 1 min recordings were made from the neurons to compare these spontaneous currents in the presence of TTX using Cs^+ filled pipettes. These spontaneous currents were present in both infected and control cultures (Figure 5) and were similar in amplitude, shape and frequency (frequency of spontaneous currents >40 pA: infected cultures 0.79 ± 0.14 Hz; control cultures 1.0 ± 0.18 Hz; n=10, respectively; P=0.29, t-test). This indicates that the postsynaptic membrane properties of cells from infected cultures are not likely deteriorated compared to controls. Furthermore, current responses to voltage steps were recorded in cells from infected cultures (no mitotic inhibitor) at day 7 p.i. without applying TTX and using pipettes filled with K^+ (140 mM) containing solutions. These cells had clear inward Na^+ and outward K^+ current responses during voltage clamp (Na^+ 4509 ± 958 pA; K^+ 494 ± 111 pA; n=8; measurements at -30 mV; series resistance compensation 60–75%) which were similar to those shown for control cells in Figure 1b (Na^+ 3060 ± 462 pA; K^+ 505 ± 107 pA, n=8; measurements at -30 mV; series resistance compensation 65–78%) and without any significant difference (Na^+ P=0.20; K^+ P=0.94; t-test). Cells from infected cultures also produced action potentials like those of Figure 1a during current clamp. Finally, the morphology of the neurons from which records were made appeared to be the same in control and infected cultures with no obvious signs of deterioration.

To determine if the intracellular Ca^{2+} levels differed between control cells and cells in infected cultures, the neurons were analysed using Ca^{2+} imaging with confocal microscopy by measuring the relative intensities of fluo-3 acetoxymethylester (fluo-3 AM). No statistical difference could be found in three series of 10 control cells and 10 cells from infected cultures 6 days p.i. (P=0.60, 0.86 and 0.40, respectively; t-test). In these experiments, both control and infected cultures were exposed to mitotic inhibitor.

We also examined the effect of the L-type Ca^{2+} channel inhibitor nifedipine and of dantrolene, which inhibits Ca^{2+} release from intracellular stores, on the survival rate of neurons at 1–7 days p.i. in infected and control cultures treated with mitotic inhibitor. Without these blockers there was a progressive loss of hippocampal neurons in infected cultures (Figure 6) starting at day 1 p.i. until day 4 p.i. when 43 ± 5% of the neurons had disappeared. During the following days p.i. there was no significant further nerve cell loss and at day 6 p.i. the nerve cell loss was 41 ± 8% (infected cultures not treated with mitotic inhibitor showed

almost a similar value, where the nerve cell loss at day 6 p.i. was $40 \pm 1\%$). In control cultures, the nerve cell loss was less than 5%.

Nifedipine was toxic to the uninfected neurons at a concentration of 100 μM, while at 50 μM no nerve cell loss was observed. No protective effect on the mumps infected neurons was seen by this drug (Figure 6a). Dantrolene had no toxic effect on normal cultures at concentrations ranging from $15-45$ μM. When tested in infected cultures, 15 μM dantrolene had no significant protective effect, while at 30 and 45 μM its neuroprotective effects were marked and significant (Mann-Whitney U-test). There was no significant loss of neurons in the infected cultures during the first 2 days p.i. and subsequently only 20% of the neurons were lost (Figure 6b). When examined at day 1 and 6 p.i., the proportion of mumps virus-infected neurons was not significantly different between cultures exposed to 30 μM dantrolene and controls ($75 \pm 2\%$ and $74 \pm 5\%$, respectively). After 6 days p.i. $61 \pm 2\%$ of the treated and $63 \pm 2\%$ of the non-treated neurons were infected.

Discussion

In the present study, inward Ca^{2+} currents in hippocampal neurons were found to be decreased in mumps virus infected primary cultures. Furthermore, the fraction of neurons that died during the infection could be reduced by treatment with dantrolene, which inhibits release of Ca^{2+} from intracellular stores.

That the reduced inward current is due to Ca^{2+} and not to an increased outward current is likely because intracellular K^+ is rapidly replaced by Cs^+ from the relatively large volume contained in the pipette. Cs^+ blocks most types of K^+ channels (Hille, 1992), whereby it is unlikely that a reduced inward current found in the study reflects a potentiation of outward current of the less permeable Cs^+-ions through K^+ channels. As the calcium channel blockers Cd^{2+} and Co^{2+} strongly reduced the inward current remaining in TTX and with Cs^+ filled pipettes, a voltage-dependent Cl^- current could not significantly contribute to the observed effects on the inward current.

Somal Ca^{2+} currents, as measured in the present study, are mediated by Ca^{2+} channels of both the L-type and the P/Q-type. The former type may be responsible for the major part of these currents because it has been reported that the selective L-type Ca^{2+} channel blockers isradipine or nimodipine inhibit 56% of the somal $[Ca^{2+}]_i$ transient following somal stimulation in CA3 neurons in organotypic cultures of rat hippocampal slices, while a combination of antagonists of P/Q-type channels block 41% of the $[Ca^{2+}]_i$ transient (Elliott *et al*, 1995). Our findings were that the reduction in infected cultures of Ca^{2+} influx was in the order of $47-55\%$ at day $6-7$ p.i. It therefore seems likely that Ca^{2+} influx through L-type channels is, if not totally, at least partially reduced by the infection. In infected cultures the Na^+ and K^+ channels responsible for action potentials responded normally during voltage clamp and the spontaneous synaptic currents were similar to those in controls. This indicates that the reduced Ca^{2+} currents observed is a selective phenomenon and not a consequence of a general disturbance of excitable membrane functions in the infected cultures.

The mechanisms behind this selective reduction in Ca^{2+} influx could be related either to virus replication in the neurons or to effects on neuronal function by infected glial cells in their environment. Virus replication within a neuron may affect its function in a number of ways. This could include a decrease in host cell macromolecular synthesis, which for paramyxoviruses can vary from an almost complete to a minimal inhibition (Lamb and Kolakofsky, 1996). Hypothetically, such a shut-off may involve host cell proteins implied in specialised functions of a neuron, such as Ca^{2+} channel proteins in the plasma membrane. Other tentative mechanisms could involve interference by viral proteins with the targeting of channel proteins to the plasma membrane, for instance, by competition during the intracellular sorting and transport of membrane proteins.

More likely, however, indirect effects on neuronal function mediated by the surrounding infected glial cells predominated in the present experiments, since reduced Ca^{2+} currents in infected cultures occurred both in neurons which were immuno-positive and -negative to the virus antigens. This was found already at days $1-2$ p.i., when a possible clearance of virus from the neurons was low. Such indirect effects could be derived from astrocytes, which are known to exert trophic influences on neurons (reviewed in Eddleston and Mucke, 1993; Patterson and Nawa, 1993). It is particularly interesting to note that neuronal maturation may be partially mediated by voltage-dependent Ca^{2+} channels, since neuronal survival and neuritic growth in chick ciliary ganglia promoted by conditioned medium from astrocytes can be inhibited by L-type Ca^{2+} channel blockers (Vaca and Wendt, 1992). In the present study, neurons in cultures which contained a higher glial cell density exhibited significantly higher Ca^{2+} currents than neurons from cultures with a lower glial cell density, i.e. those exposed to the mitotic inhibitor. A trophic influence by astrocytes on neurons may therefore have been disturbed by the mumps virus infection. Thus, it is suggested that a disturbance in the interactions between glial cells and neurons may cause the observed decrease in voltage-dependent Ca^{2+} currents in the neurons.

An altered influx through the somal voltage-dependent Ca^{2+} channels may influence the survival of the neurons. An excessive Ca^{2+} influx has, in general, been considered to be associated with nerve cell death and an increase in the number of L-type channels has been proposed to be involved in neurodegenerative conditions (Wagner *et al*, 1986) and in the enhanced vulnerability of hippocampal neurons during ageing (Thibault and Landfield, 1996). Consequently, a reduced Ca^{2+} influx, as observed in our mumps virus infected cultures, and treatment with Ca^{2+} channels blockers, might be expected to have neuroprotective effects. However, in contrast to such protective effects by L-type channel blockers observed previously during HIV-1 infections (Dreyer *et al*, 1990) and in mumps virus infected dorsal root ganglia neurons (Andersson *et al*, 1991), the present attempts to rescue neurons by the L-type Ca^{2+} channel blocker nifedipine were unsuccessful. This may indicate either that further reduction of the already reduced L-type Ca^{2+} currents in infected cultures had no effect on neuronal survival or that Ca^{2+} influx through these channels did not contribute substantially to the nerve cell death in our experiments. A number of studies have, indeed, shown that blockers of voltage-dependent Ca^{2+} channels may fail to prevent early neurotoxicity (Madden *et al*, 1990; Weiss *et al*, 1990) and that Ca^{2+} influx through these channels can even promote neuronal survival and differentiation (Gallo *et al*, 1987; Collins *et al*, 1991). For example, L-type Ca^{2+} channel antagonists block depolarisation-enhanced survival of rat sympathetic and cerebellar granule neurons (reviewed in Franklin and Johnson, 1992), and spontaneous voltage-dependent Ca^{2+} spikes seem to promote differentiation of embryonic neurons (reviewed in Spitzer, 1994). The cyclic AMP-responsive element binding protein (CREB) pathway have been implicated in the Ca^{2+} signalling of gene expression (Bito *et al*, 1996). Since votage-dependent Ca^{2+} currents in DRG neurons are of a higher magnitude, and since DRG neurons have a relatively low capacity to buffer $[Ca^{2+}]_i$, differences in the effect of blockers of these channels on neuronal survival in infected cultures of these two types of cells, as we have observed, can be anticipated (Jia and Nelson, 1986).

While effects of Ca^{2+} influx through L-type Ca^{2+} channels may vary, Ca^{2+}-mediated neurotoxicity seems mainly to be related to influx through the NMDA receptors channels (Choi, 1988; Tymianski *et al*, 1993). Such observations have indicated that the effects of Ca^{2+} influx on the neuron depend on the route of its entry and that signals elicited by Ca^{2+} may be compartmentalised (Tymianski *et al*, 1993; reviewed in Ghosh and Greenberg, 1995). In the present study, a neuroprotective effect was observed after exposure of the infected cultures to dantrolene. This drug inhibits release of Ca^{2+} from intracellular stores in different cellular types and it has been shown that in neurons dantrolene can inhibit 70% of glutamate-induced increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} and the whole increase in $[Ca^{2+}]_i$ in its absence (Frandsen and Schousboe, 1991). The protective effect of dantrolene on mumps virus-infected neurons, therefore, indicates that virus-induced lysis of hippocampal neurons (which amounted to about 40% of the neurons) to a large extent is mediated by release of Ca^{2+} from intracellular stores. As shown by confocal microscopy there was no difference in $[Ca^{2+}]_i$ in neurons from control and infected cultures 6 days p.i. An increase in neuronal $[Ca^{2+}]_i$ in infected cultures may, however, have occurred earlier during infection, at a time when neurons were lost, either as sustained high concentrations during that period or as transient oscillations (Lannuzel *et al*, 1995). Since both uninfected neurons, as well as infected ones, were rescued to a similar extent by dantrolene, it is likely that a toxic release of Ca^{2+} from intracellular stores was caused by stimuli from the surrounding cells. Such stimuli may, for instance, be related to excitatory amino acids which either induce Ca^{2+} influx through NMDA channels to stimulate ryanodine receptor-activated Ca^{2+} release from intracellular stores (McPherson *et al*, 1991; reviewed in Mody and MacDonald, 1995) or to activation of metabotrophic glutamate receptors to cause Ca^{2+} release from the intracellular stores through the inositol triphosphate pathway (reviewed in Ghosh and Greenberg, 1995). Enhanced levels of excitatory amino acids in the medium of infected cultures may have derived either from a reduced uptake of glutamate in infected astrocytes (cf Rothstein *et al*, 1996) or from release of quinolinic acid from activated glial cells (Köhler *et al*, 1988; Lipton, 1994).

In addition to the proposed effects of glial cells on the neuronal Ca^{2+} homeostasis in the infected cultures, an effect of these cells on the expression of viral antigens in neurons may be suggested. Thus, cultures not treated with mitotic inhibitor displayed a substantial reduction in the number of neurons immuno-positive for viral antigens between 1–2 and 6–7 days p.i., while cultures exposed to mitotic inhibitors did not. Since the former cultures displayed a higher density of glial cells than the latter, it is possible that the glial cells can affect the metabolism or maturation of the neurons so that a clearance of the viral infection from the cytoplasm is favoured (cf Löve *et al*, 1987). It is well known that the susceptibility of neurons to a viral infection varies with their degree of differentiation (Griffin *et al*, 1994; Levine *et al*, 1996).

In conclusion, the present results show that mumps virus can have selective effects on somal voltage-dependent Ca^{2+} currents in hippocampal neurons. These Ca^{2+} currents are involved in the

regulation of shape and frequency of action potentials by activating K^+ and Cl^- channels. They are also involved in the activation of Ca^{2+}-dependent enzymes and in gene expression by signalling to the nucleus. Together with studies showing that changes in selective synaptic functions in the brain may persist after a viral infection (Pearce *et al*, 1996) and that persistently infected neurons may display depressed neurotransmitter mRNA levels (Lipkin *et al*, 1988), these findings indicate that virus infections during early life may cause selective disturbances in the development of differentiated neuronal functions in the brain.

Materials and methods

Cell culture

Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden) on the 18th gestational day, kept under standard laboratory conditions, were killed using carbon dioxide and the hippocampi dissected from the foetuses. Cultures were then prepared according to Rothman (1984). The dissected hippocampi were incubated at 37°C for 15 min in 0.1% trypsin (Gibco, Paisley, Scotland) diluted in Ca^{2+}-Mg^{2+}-free Hank's Balanced Salt Solution (pH 7.3) and subsequently triturated through a narrowed Pasteur pipette. The cell suspension was then seeded into 35×10 w/2 mm Grid Tissue Culture Dish (Nunc, Inc., Naperville, IL) at a density of approximately 500 000 cells per dish, which had been precoated with poly-L-lysine hydrobromide (MW $3-7 \times 10^4$, Sigma Chemical Company, St Louis, MO).

The cells were grown in 2 ml of Dulbecco's Modified Eagle's Medium/Ham's F12 (50/50, Gibco) with the following additives: 1.2 mg/ml glucose (BDH, Poole, UK), 10% foetal calf serum, 5 μg/ml bovine insulin, 100 μg/ml human transferrin, 20 units-μg/ml penicillin/streptomycin (all from Gibco), 20 nM progesterone, 100 μM putrescine and 30 nM selenium dioxide (all from Sigma). Established cultures were maintained in an incubator providing 5% CO_2 at 37°C. In order to inhibit division of non-neuronal cells in the cultures, 5-fluorodeoxyuridine (Sigma) was added after 6 days, before glial cells had reached confluentic growth, at a concentration of 15 μg/ml to half of the culture dishes, whereas in the other half of the dishes no mitotic inhibitor was added. The growth medium was not changed and no refeeding was done.

On the 14th day in culture, the cultures were infected with mumps virus (RW-strain, kindly provided by Dr. Jerry Wolinsky, University of Texas Health Science Center at Houston, Houston, TX) at a titre of 2×10^7 plaque forming units per ml.

Electrophysiology

Whole cell patch clamp recordings (Hamill *et al*, 1981) were made on cells which were selected using an inverted phase contrast microscope (Nikon Diaphot 300). Cells were chosen according to a strict morphological criterion of having a roughly triangular shape with conspicuous processes, and which were similar in size, having a soma width of $15-20$ μm. Subpopulations of cells with this morphology were quite distinct and had similarities to hippocampal pyramidal neurons. Cells with irregular branching patterns or growing relatively isolated from the rest of the subpopulation were avoided. For experiments with mumps virus, measurements were made on alternating control and infected culture dishes of cells which otherwise had identical incubation times and treatment. Control cells were compared with infected cells at days $1-2$ and $6-7$ p.i. Recordings were made at room temperature. Culture medium was replaced with a physiological solution consisting of the following ingredients (in mM): NaCl, 140; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; sucrose, 10; *N*-[2-hydroxyethyl]-piperazine-N′-[2-ethanesulphonic acid] (HEPES), 10. The solution was brought to pH 7.40 with NaOH and osmolarity adjusted to 305 ± 2 mOsm with sucrose. A pumping system was used to exchange solutions which also contained, depending on the experiment, tetrodotoxin (TTX), Co^{2+}, Cd^{2+}, 2-amino-5-phosphonovalerate (APV; all from Sigma) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Neuramin, Bristol, UK).

Current and voltage clamp records were obtained using an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc., Foster City, CA). Patch pipettes were pulled to have a resitance of $3-7$ MΩ and were filled with one of two solutions having the following ingredients (in mM): solution 1, CsCl, 140; NaCl, 4; $CaCl_2$, 0.5; $MgCl_2$, 1; ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5; HEPES, 10; buffered to pH 7.40 with CsOH; solution 2, KCl, 140; NaCl, 4; $CaCl_2$, 0.5; $MgCl_2$, 1; EGTA, 5; HEPES, 10; buffered to pH 7.40 with KOH. Seals with over 1 GΩ resistance were made by applying slight suction and negative voltage after contact with the cells. The validity of adequate seals between the pipette and cell membrane, without undue intra-pipette membrane distortion, was assured by requiring observation of single ion channel currents prior to membrane rupture. Upon rupture during successful whole cell patches, a sudden increase occurred in the time constant of current transients at onset and offset of a 1 mV negative step. This time constant was monitored often during whole cell recordings to check that access to the cell's interior was not diminished; if so, a small amount of suction was reapplied to reopen the pipette tip. Because of the primary focus of this study, where the interest was the maximum observable inward Ca^{2+} current within a broad voltage range, the error between pipette and actual membrane potential was not of significance and therefore series resitance was not always compen-

sated. The summed access and membrane input resistances at −60 mV were between 200 and 500 MΩ and typical pipette resistance after removal from the cell was 20 MΩ. Command voltages, stimulus current pulses and digitised data sampling were controlled by an A/D, D/A and timing computer interface (TL-1; Axon Instruments, Inc.) in conjunction with PC software (pClamp and Axotape, Axon Instruments, Inc.). Leakage currents were subtracted online by the P/N protocol using four negative prepulses or by leak resistance compensation during analysis (pClamp software, Axon Instruments, Inc.).

Immunohistochemistry

Immediately following electrophysiological experiments, cultures were washed in 0.01 M phosphate buffered saline (PBS; pH 7.4), dried for 2 h in 37°C, fixed in cold (4°C) 4%-paraformaldehyde in PBS for 30 min and then rinsed in PBS. Immunohistochemical staining was then performed in order to examine whether the neurons from which successful voltage clamp records had been made were infected. These cells where marked at the bottom of the culture dish immediately after recording by braking the tip of the glass electrode pipette at 1–4 places in the vicinity of the cell, making the later identification of each cell exact. Cultures were preincubated with 2% normal swine serum (DAKO A/S, Glostrup, Denmark) in PBS with 0.3% Triton X-100 added (Eastman Kodak Company, Rochester, NY) for 30 min at room temperature. The cells were then incubated overnight at 4°C with a rabbit polyclonal antibody against mumps virus (K122), diluted 1:500 in PBS containing 2% normal swine serum and 0.3% Triton X-100. After washing in PBS, the cultures were incubated with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit antibodies (DAKO), diluted 1:30 in PBS with 2% normal rat serum (DAKO), for 30 min at 37°C, washed in distilled water and mounted in PBS-buffered glycerine with 0.1% p-phenylenediamine (Sigma). Uninfected cultures were processed as described above to serve as controls.

The proportion of neurons containing viral antigen was determined by double-immunolabelling in five infected cultures at days 1 and 6 p.i., respectively, both in cultures grown with or without mitotic inhibitor. After fixation and preincubation with 5% normal swine serum (DAKO), the cells were incubated with the rabbit polyclonal antibody against mumps virus (K122) for 60 min at 37°C. The cultures were then rinsed in PBS and subsequently incubated overnight at 4°C with a mouse monoclonal antibody to microtubule-associated protein 2 (MAP2; Sigma) diluted 1:800 in PBS with 2% normal horse serum (Vector Laboratories, Burlingame, CA) and 0.3% Triton X-100 added. After exposure to the second antibody, the cultures were rinsed in PBS and incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine anti-rabbit antibodies (DAKO) in PBS with 2% normal rat serum for 30 min at 37°C. The cultures were then rinsed in PBS and finally incubated with FITC-labelled rabbit anti-mouse antibodies (DAKO) in PBS with 2% normal rat serum for 30 min at 37°C, rinsed in distilled water and mounted in PBS-buffered glycerine with 0.1% p-phenylenediamine. Uninfected cultures that had been processed as described above served as controls.

Astrocytes were examined immunohistochemically with antibodes against glial fibrillary acidic protein (GFAP). After fixation and preincubation with 5% BSA (Sigma) in PBS for 30 min, the cultures were incubated with a rabbit polyclonal antibody against cow GFAP (DAKO), diluted 1:1000 in 5% BSA in PBS with 0.3% Triton X-100 added, and kept overnight at 4°C. After washing in PBS, the cultures were incubated with FITC-labelled swine anti-rabbit antibodies (DAKO) diluted 1:30 in PBS with 2% normal rat serum (DAKO) for 30 min at 37°C, rinsed in distilled water and mounted as above. Visualisation of the immunolabelling was done in an epifluorescence microscope.

Confocal microscopy

Cell cultures were loaded with 5 μM fluo-3 acetoxymethylester (Molecular Probes Europe BV, Leiden, The Netherlands) for 30 min at 37°C. Cells were then washed twice and kept in a physiological solution during the measurements, which were made at 20°C. The images of the relative fluorescence from fluo-3 AM were collected using a Noran Odyssey XL confocal microscope.

Neuronal survival after nifedipine and dantrolene exposure

After 14 days, the cultures were infected with mumps virus described as above. Virus-infected cultures were exposed to either dantrolene (15, 30 or 45 μM; Sigma) dissolved in distilled water or nifedipine (50 or 100 μM; Sigma) dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) immediately after virus infection. The effects on neuronal survival were compared with infected but untreated cultures. As controls served also uninfected-untreated cultures and uninfected cultures exposed to either dantrolene, nifedipine or DMSO. For calculations of neuronal survival, 2–3 areas, each containing 100–300 neurons before infection, were defined and marked on the culture dishes. The dishes were coded and the neurons were counted daily in a phase-contrast microscope. Six separate series of experiments were performed employing 3–5 culture dishes for each group.

Statistical analysis
Values are given as mean ± standard error of the mean (s.e.m.). Data were analysed by Student's *t*-test, one-way analysis of variance (one-way ANOVA) followed by Dunnett's *t*-test post-hoc comparison, or by Mann-Whitney U-test, as appropriate.

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