Cerebrospinal fluid from multiple sclerosis patients inactivates neuronal Na\(^+\) current

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Summary

Multiple sclerosis is a common inflammatory disease of the CNS. A great number of immunologically active molecules have been identified in the CSF of these patients (CSF-MS), but the role of these substances in neuronal dysfunction, especially in the origin of transient symptoms, is unclear. Therefore, we investigated the effect of CSF from 13 multiple sclerosis patients on membrane currents of cultured cortical neurons from embryonic rat and compared it with the effect of CSF from 12 patients with non-inflammatory neurological diseases. We found an increase in Na\(^+\) current (I\(_{\text{Na}}\)) inactivation by a shift of the h\(_{\infty}\) curve to more hyperpolarizing potentials by 9.3 mV. This effect was reversible by washing and could be abolished by CSF-MS heat inactivation. The degree of the shift ranged from 4.3 mV to 17.6 mV and correlated with the IgG index, but not with the degree of pleocytosis, protein or albumin content. The maximal amplitude of I\(_{\text{Na}}\) was unchanged. We concluded that diffusible factors are released into the CSF which reduce neuronal excitability and thereby disturb the function of the neuronal network. These factors may well contribute to transient neurological symptoms seen in patients with "active" multiple sclerosis.

Keywords: multiple sclerosis; sodium current; neuronal cell culture; cerebrospinal fluid; cytokines

Abbreviations: CTRL = control; Ig = immunoglobulin; IL = interleukin; I\(_{\text{A}}\) = potassium A current; I\(_{\text{K}}\) = delayed potassium outward current; I\(_{\text{Na}}\) = sodium current; TNF = tumour necrosis factor; V\(_{\text{H}}\) = holding potential; V\(_{1/2}\) = half maximal voltage

Introduction

Multiple sclerosis is an inflammatory disease of the CNS with focal symptoms such as optic neuritis, mainly due to local demyelination, and global symptoms such as cognitive impairment (Thompson and McDonald, 1992; McDonald, 1993). Since the work of Bornstein and Crain (1965), whether factors, which impair neuronal excitability are secreted into serum or CSF has remained controversial. Schauf et al. (1976, 1978, 1981) found a depression of polysynaptic activity in isolated spinal cord of the frog after incubation with sera from multiple sclerosis patients. Seil et al. (1976, 1977), however, denied a specific depression of neuroelectric activity by sera from multiple sclerosis patients. They suggested, that as a result of increased permeability of the blood–brain barrier in inflammation, factors normally present in serum enter the CNS, thus impairing neuronal excitability. Recently, Brinkmeier et al. (1993) reported a decrease of Na\(^+\) currents in human myoballs induced by CSF from multiple sclerosis patients.

A number of cytokines including interleukin (IL) 1\(\beta\) (Hauser et al., 1990; Tsukada et al., 1991), IL-2 (Gallo et al., 1988, 1991), IL-6 (Maimone et al., 1991; Weller et al., 1991) and tumour necrosis factor (TNF)-\(\alpha\) (Franciotta et al., 1989; Hauser et al., 1990; Sharief and Hentges, 1991; Tsukada et al., 1991) have been identified in CSF of patients with acute multiple sclerosis. In multiple sclerosis plaques, the presence of cytokines, e.g. IL-1 (Wucherpfennig et al., 1992) and TNF (Hofman et al., 1989; Cannella and Raine, 1995) has been detected. The relevance of such immune mediators for the immunopathological process is currently under investigation (Hartung et al., 1992; Hartung, 1993). In addition, immunologically active molecules can affect electrophysiological properties of neurons either directly (Sawada et al., 1990, 1991; Köll er and Siebler, 1993; Plata-Salaman and ffrench-Mullen, 1993) or indirectly, mediated by an alteration of the electrophysiological functions of immunologically activated astrocytes (Köller et al., 1993, 1994a, b).

The aims of the present study were (i) to investigate...
Cell culture methods were the same as described previously (Stichel and Müller, 1992; Köller and Siebler, 1993). Briefly, neurons were prepared from cortices of embryonic Wistar rats at embryonic day 15. After short trypsinization and gentle trituration with fire polished Pasteur pipettes, the cell suspension was passed through a 33-μm gauze and washed by centrifugation. Cells were plated on glass coverslips, which were previously coated by poly-l-lysine (0.1 mg ml⁻¹, 24 h, 4°C) and laminin (10 μg ml⁻¹, 24 h, 4°C) at densities of 6–10×10⁵ cells/cover slip. They were grown in serum-free supplemented N2 media (Bottenstein and Sato, 1979; Stichel and Müller, 1992), conditioned by a spatially separated monolayer of cerebral astrocytes for at least 3 days prior to transferring to neuronal cultures. Cells were identified as neurons by electrophysiological and by immunohistochemical methods (Stichel and Müller, 1992), and no glial fibrillary acidic protein (GFAP) positive cells were found in neuronal cultures. Electrophysiological recordings were performed on cortical neurons between 5 and 12 days in vitro.

### Material and methods

**CSF**

CSF was obtained from 15 multiple sclerosis patients (seven males and eight females, aged 18–49 years) who were admitted to our department during an acute relapse. According to the criteria of Poser et al. (1983), these patients had either clinically definitive multiple sclerosis (n = 11) or laboratory supported definitive multiple sclerosis (n = 4). Oligoclonal bands were positive in all CSF-MS, IgG indices (IgG in CSF/IgG in serum divided by albumin in CSF/albumin in serum; normal values below 0.7) were elevated in 11 cases. Control CSF (CSF-CTRL) was obtained from 12 patients (seven males and five females, aged 20–65 years) with one of the following symptoms or diagnoses: headache in absence of meningitis, encephalitis or haemorrhage (seven patients), normal pressure hydrocephalus (three), spondylotic myelopathy (one), brain tumour (one). Cell counts and IgG index were normal in every CSF-CTRL. Oligoclonal bands were evaluated in each case and found to be negative. Values of protein content, albumin content, cell count and IgG index are given in Table 1. Between 1 and 2 ml of each CSF sample were centrifuged immediately after lumbar puncture and stored at −70°C until use for electrophysiological experiments. All patients consented to the use of 1 or 2 ml CSF for scientific purposes and experiments had the local ethical committee approval.

### Cell culture

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### Electrophysiological recordings

Electrophysiological recordings were performed by means of the patch clamp technique (Hamill et al., 1981) in the whole-cell recording configuration using a patch clamp amplifier (EPC 7, List, Germany). Prior to electrophysiological recording, cells were transferred to CSF-MS, CSF-CTRL or bath solution, the latter containing (mM): NaCl (150.0), KCl (4.0), CaCl₂ (2.8), MgCl₂ (1.0), HEPES (10.0), sucrose (10.0); pH adjusted to 7.4 by NaOH. Recording electrodes had tip diameters of ~1 μm and resistances of 5–6 MΩ. Pipettes were drawn from borosilicate glass capillaries without filament (GC 150–15, Clark Electromedical Instruments, Pangbourne, UK) and were filled with a solution of (mM): KCl (140.0), CaCl₂ (1.0), MgCl₂ (2.0), EGTA (11.0), HEPES (10.0); pH adjusted to 7.4 by KOH. In order to further analyse Na⁺ currents, we performed a number of experiments with a K⁺-free intracellular pipette solution. KCl in these experiments was replaced by CsCl and tetraethylammoniumchloride (10 mM) was added to block K⁺ channels. The recording chamber had a volume of 1 ml. Wash procedures from CSF-MS were performed by transferring the cultures into bath solution for several minutes and than transferring to CSF-CTRL. Substances like cytokines were added to the bath solution to achieve the desired concentrations. Recordings were done at room temperature.

### Table 1 CSF parameters for CSF-MS and CSF-CTRL

<table>
<thead>
<tr>
<th></th>
<th>CSF-CTRL (n = 12)</th>
<th>CSF-MS (n = 13)</th>
</tr>
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<tbody>
<tr>
<td>Cell count (μl⁻¹)</td>
<td>1.4±0.8 (1–3)</td>
<td>16.3±15.0* (1–49)</td>
</tr>
<tr>
<td>Protein content (g l⁻¹)</td>
<td>0.52±0.28 (0.20–1.15)</td>
<td>0.47±0.25 (0.18–1.06)</td>
</tr>
<tr>
<td>Albumin content (mg l⁻¹)</td>
<td>331±193 (109–737)</td>
<td>232±107 (98–460)</td>
</tr>
<tr>
<td>IgG index</td>
<td>0.48±0.05* (0.40–0.57)</td>
<td>1.13±0.44* (0.62–2.11)</td>
</tr>
<tr>
<td>Oligoclonal bands</td>
<td>Negative (n = 12)</td>
<td>Positive (n = 13)</td>
</tr>
</tbody>
</table>

The range is given in parenthesis.*Significant difference between CSF-CTRL and CSF-MS according to P < 0.001; Student's t test.
Interleukin 1β and TNF were purchased from Genzyme (Boston, Mass., USA); IL-6 from Pharma Biotechnologie (Hannover, Germany); all other chemical substances used were purchased from Sigma (Deisenhofen, Germany).

Membrane potentials were measured immediately after establishing the whole cell configuration and again after at least 5 min, with the exception of those cells which were investigated with K+ -free intracellular solutions. Cells showing marked changes of membrane potential within this time were excluded from further study. Membrane resistances were evaluated by applying depolarizing and hyperpolarizing current pulses from a holding potential of 80 to 100, 90, 70 or 60 mV and calculated as the inverse of the slope conductance between 100 and 70 mV. The calculation of the resistance served as control to exclude major cell damage by the recording pipette and to exclude cells for which a loss of recording quality occurred during the time of experiment. Neurons with resistances below 300 MΩ were excluded.

Na+ currents were elicited by intracellular current pulses (50 ms) which were applied from various holding potentials (V_H) beginning with a V_H of -110 mV to clamp the membrane to potentials of -40 mV. After ~5 s, the V_H was manually adjusted to the next V_H tested (-100 mV) and the current pulse was applied again to clamp the membrane to a potential of -40 mV. The same procedure was repeated in steps of 10 mV for V_H from -110 mV to -50 mV. Depolarization pulses from V_H more negative than -110 mV did not lead to an increase of the elicited Na+ current. Na+ current (I_Na) inactivation was estimated as follows: normalized inward currents were calculated by dividing the magnitude of the peak inward current elicited from various V_H by the magnitude of peak inward current elicited from the V_H of -110 mV, both currents corrected for the leakage current. Late outward currents, regarded as the delayed K+ outward current (I_k) were measured 300 ms after pulses began (after subtracting the leakage current). The fast activating-fast inactivating outward current, resembling the potassium A current (I_h) was evaluated by subtracting the outward current at 300 ms from the maximal outward current.

All recordings were filtered at 5 kHz, digitized with a sampling rate of 10 kHz, stored and analysed on a digital oscilloscope (Nicolet 4049) and a personal computer using software written in our laboratory. Activation and inactivation curves were estimated for each single cell by fitting the data to the Boltzmann equation (Sigma plot, Jandel Corte Madera, USA). The estimated half maximal voltage (V_1/2) values obtained from groups of neurons in CSF from different patients were compared using Student’s t test. Statistical data are presented as means and standard deviations.

### Results

In all, 336 neurons were tested using 25 CSF samples, 13 from patients with an acute relapse of multiple sclerosis (CSF-MS) and 12 from control patients (CSF-CTRL) with noninflammatory neurological diseases.

**Fig. 1** CSF-MS blocked Na+ inward current after depolarization from low holding potentials. Cortical neurons were depolarized to -40 mV by intracellular current application from holding potentials as indicated (V_H; see inset for pulse protocol). In CSF-CTRL most cells showed a Na+ inward current, when cells were depolarized from -70 mV as well as from -100 mV. In CSF-MS Na+ inward currents were blocked at a holding potential of -70 mV, whereas from -100 mV an inward current was elicited. There were no significant differences in the magnitude of Na+ inward currents elicited from V_H of 100 mV. Dotted lines represent 0 current injection.

All cells were identified as neurons either by eliciting an action potential or a rapidly activating and inactivation inward current upon depolarization (Ahmed, 1988a). In CSF-CTRL, we tested the sensitivity of the inward current to tetrodotoxin (TTX, 10 µM), which completely blocked inward currents in each cell tested (n = 8). The membrane potential and membrane resistance were similar in CSF-MS and CSF-CTRL. In CSF-MS, the membrane potential was measured as -40.0±7.0 mV (n = 82 cells), in CSF-CTRL as -36.0±9.9 mV (n = 52). Membrane resistances were 908±295 MΩ (in CSF-MS) and 998±550 MΩ (in CSF-CTRL), respectively.

**Effect on Na+ inward current**

The maximal I_Na, elicited by depolarizing pulses from a holding potential of -100 mV to -40 mV were similar in both groups: 1.098±0.5 nA (n = 20) in CSF-MS and 1.136±0.6 nA (n = 10) in CSF-CTRL. The I_Na inactivation, however, differed markedly. A significantly higher portion of neurons in CSF-MS showed a complete inactivation of Na+ currents when inward currents were elicited from holding potentials of -70 and -80 mV (Fig. 1).

The Na+ current inactivation as well as the activation curve was described by a datum fit to the Boltzmann equation:

\[ h_x(V) = \frac{1}{1 + \exp \left( \frac{V_m - V_{1/2}}{k} \right)} \]

where \( h_x \) is the fraction of channels inactivated, \( V_m \) represents the holding potential. The slope factor \( k \) did not differ significantly
The Na⁺ current inactivation curve was shifted by CSF-MS; this shift could be abolished by heat inactivation. The normalized $I_{Na}$ ($I_{Na}$ from various $V_h$ divided by the maximal $I_{Na}$, called 'Na⁺ current norm' in the diagram) was shown. Data were fitted to the Boltzmann equation. Symbols represent mean values with symmetrical error bars, shown in the positive direction for recordings in CSF-CTRL (open circles) and in the negative direction for CSF-MS (closed circles). There were significant differences in the $V_{1/2}$ values in the inactivation curve as well as for the portion of Na⁺ currents elicited from $V_h$ of -80 mV and -70 mV between recordings in CSF-CTRL and CSF-MS ($P < 0.001$). The shift between the Na⁺ current inactivation curve in CSF-CTRL and CSF-MS was reversible after repeated wash procedure in saline solution. Recordings were then performed in CSF-CTRL (open squares, data fit not shown). After heat inactivation of CSF-MS by heating to +56°C for 45 min (filled triangles, dotted line) no shift of the inactivation curve was observed.

between CSF-MS and CSF-CTRL (CSF-MS: 6.20±0.45 mV versus CSF-CTRL: 7.34±1.63 mV). $V_{1/2}$ was significantly different in CSF-CTRL: $V_{1/2}$ was -82.3±5.7 mV ($n=82$), whereas in CSF-CTRL $V_{1/2}$ was -71.6±5.6 mV ($n=52$; Fig. 2, $P < 0.001$). The estimated mean values and SDs of $V_{1/2}$ for recording in the various CSF-CTRL and CSF-MS are given in Table 2. There was no significant difference in the $I_{Na}$ activation curve: $V_{1/2}$ was measured as -54.0±4.1 mV in CSF-MS and -58.2±1.1 mV in CSF-CTRL ($n=11$; data not shown). The shift of the $I_{Na}$ inactivation curve was reversible: after washing the coverslip in ionic bath solution and recording of sibling neurons afterwards in CSF-CTRL, $V_{1/2}$ was -74.8±1.1 mV ($n=9$; Fig. 2).

We tested whether the shift of the $I_{Na}$ inactivation curve was induced by a heat sensitive factor and performed a heat inactivation of CSF-MS at +56°C for 45 min. After heat inactivation, the shift was abolished: $V_{1/2}$ was -73.0±1.39 mV ($n=16$), not significantly different from the control values given in Fig. 2. The slope factor $k$ was unchanged: 8.1±1.24 mV.

The $V_{1/2}$ of the $I_{Na}$ inactivation curve in CSF-MS ranged from -77.2±5.8 mV to -90.5±3.1 mV for different CSF samples from different multiple sclerosis patients. We tested the linear correlation of the inactivation shift with CSF parameters. There was a correlation with the IgG index ($r = 0.13$) or albumin ($r = 0.01$) content (data not shown).

K⁺ currents are not affected by CSF-MS

In most cells, two types of K⁺ currents could be elicited in CSF-CTRL as well as in CSF-MS: a slowly activating and
Various cytokines have been identified in the CSF of multiple sclerosis patients. We tested whether the effect of any of these cytokines mimicked the effect of CSF-MS on the Na\(^+\) current inactivation curve. Added to the bath solution, neither IL-1β (500 or 1000 U ml\(^{-1}\); n = 24), IL-2 (500 U ml\(^{-1}\); n = 14), IL-6 (1000 U ml\(^{-1}\); n = 24) nor TNF-α (1000 U ml\(^{-1}\); n = 26) shifted the inactivation curve, compared with recordings in ionic bath solution (n = 33) without cytokines (Fig. 4).

**Cytokines did not increase I\(_{Na}\) inactivation**

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**Discussion**

The main results of the present study are that CSF of multiple sclerosis patients shifted the Na\(^+\) current inactivation curve to hyperpolarized potentials by 9.3 mV without affecting the slope factor or the magnitude of I\(_{Na}\). This shift correlated positively to the increase of IgG index, but not to the cell counts in CSF or the protein content. The effect of the CSF-MS could not be explained by the action of one of the cytokines IL-1β, IL-2, IL-6 or TNF-α, which did not induce an increase of Na\(^+\) current inactivation.

The voltage dependent Na\(^+\) current can be blocked by direct binding of a drug to the channel, as shown for TTX, by lipophilic interaction as presumed for local anaesthetics (Hille, 1992; Ragsdale et al., 1994), or by processes involving second messenger systems (Numann et al., 1991). Anticonvulsants such as valproate also shift Na\(^+\) current inactivation curves towards hyperpolarized potentials (Van den Berg et al., 1993).

Besides these drugs, serum components have also been reported to shift the Na\(^+\) current inactivation curve. Zubov et al. (1991) reported that in a neuroblastoma cell line voltage-dependent Na\(^+\) current inactivation and activation are affected by serum components, and they identified albumins as the active compound. CSF from patients with inflammatory diseases has also been shown to affect Na\(^+\) currents. Brinkmeier et al. (1993) reported that CSF from multiple sclerosis patients induced a 25–75% decrease in Na\(^+\) currents in human myoballs. They also observed a shift of the Na\(^+\) current inactivation curve towards more negative potentials. Similar results have been obtained for Na\(^+\) currents in human myoballs measured in CSF from patients suffering from the Guillain–Barré syndrome (Brinkmeier et al., 1992).

Cytokines have been identified in CSF of patients with a number of neurological diseases (Gallo et al., 1988, 1991; Franciotta et al., 1989; Hauser et al., 1990; Maimone et al., 1991; Sharief and Hentges, 1991; Tsukada et al., 1991; Weller et al., 1991). Their effects on membrane currents have been investigated and a decrease of voltage-dependent Na\(^+\) and K\(^+\) currents induced by IL-2 and TNF has been described: Kaspar et al. (1994) found a blocking effect of IL-2 in concentration of 500 U ml\(^{-1}\) on the I\(_{Na}\) of human myoballs. Tumour necrosis factor (180–900 U ml\(^{-1}\)) induces a decrease in both Na\(^+\) conductance (Sawada et al., 1991) and in K\(^+\) conductance (Sawada et al., 1990) in aplysia neurons. In acutely isolated hippocampal neurons, IL-2 depresses Ca\(^2+\) currents (Plata-Salamán and French-Mullen, 1993). Therefore, we tested whether one of these cytokines is able to mimic the effect on Na\(^+\) currents induced by CSF-MS. No effect of IL-1β, IL-2, IL-6 or TNF was observed in our model.

The nature of the blocking factor is unknown. It would appear to be a heat sensitive protein, since the inactivating effect of the CSF-MS could be abolished by heating. Differences between both groups of CSFs concerning pH or ionic composition seem to be very unlikely, since samples...
were processed identically, and ion composition should not be changed by heating. Due to limited amounts of CSF available, we were not able to control each of these parameters. Comparing CSF parameters from both groups obtained from routine diagnostic procedures, no differences occurred in protein or albumin content, but there was a major difference in IgG index. The elevation of intrathecal IgG synthesis is a usual finding in multiple sclerosis and its elevation in the CSF is used diagnostically and belongs to the paraclinical parameters for multiple sclerosis according to Poser et al. (1983). A correlation with disease activity, however, has not been shown. The moderate correlation of the shift of the Na\(^+\) current inactivation curve to the IgG index suggests that the blocking activity is associated with the intrathecal immunological process. The induction of blocking activity by serum components, which enter the CSF after breakdown of the blood–brain barrier, as proposed by Seil et al. (1976), seems to be unlikely.

What are the consequences of this increased Na\(^+\) current inactivation? The shift of the Na\(^+\) current inactivation curve to hyperpolarized potentials will decrease the excitability not only of individual single neurons, but also of local networks putatively in areas neighbouring inflammatory lesions. Secondly, assuming that mechanisms of Na\(^+\) channel inactivation similar to those described for peripheral nerves (Rasminsky and Sears, 1972; Yokota et al., 1994) also apply to the CNS, increased Na\(^+\) channel inactivation can induce conduction block and conduction slowing. Reduction of inflammation with a decrease in blocking activity may allow improvement much faster than could be expected by remyelinating processes. Youl et al. (1991) investigated the correlation between gadolinium-diethylene triamine penta-acetic acid (Gd-DTPA) leakage in optic nerve MRI as a marker of local inflammation and electrophysiological findings in acute optic neuritis. In symptomatic nerves, which displayed Gd-DTPA leakage, they observed a reduction of amplitude and an increase in latency in evoked potentials. A reduction of Gd-DTPA leakage in the re-examination 4 weeks later was associated with a significant increase in visual evoked potential amplitude. The authors concluded that a conduction block was induced by the local inflammatory process, which accounted for the clinical deficits and the resolution of the conduction block may initiate clinical recovery. Caution is always necessary in extrapolating from animal cell culture data to effects in human diseases, but we suggest that increased sodium current inactivation, as described here, may represent the pathophysiological origin of the clinically observed conduction block.

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