A Relative Energy Failure Is Associated With Low-Mg\(^{2+}\) But Not With 4-Aminopyridine Induced Seizure-Like Events in Entorhinal Cortex

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INTRODUCTION

In the early phases of status epilepticus, this potentially deleterious condition is treatable in the majority of cases with standard anticonvulsants. However, pharmacological control becomes increasingly difficult the longer the condition has endured (Shorvon 1994). An energetic failure of the epileptic neurons has been suggested to be a major factor in the transition from treatable to refractory status epilepticus (Treimann et al. 1989).

Depolarization of neurons during epileptiform activity promotes Ca\(^{2+}\) influx through voltage-activated and N-methyl-D-aspartate (NMDA)-gated ion channels, raising the [Ca\(^{2+}\)] (Uematsu et al. 1990; van den Pol et al. 1996). Such elevations in [Ca\(^{2+}\)], result in a depolarization of mitochondrial membranes and in subsequent increases in intramitochondrial Ca\(^{2+}\) concentration (Duchen 1992). As a consequence, respiration is increased and the concentration of reactive oxygen species elevated (Dugan et al. 1995). Furthermore, increased intramitochondrial Ca\(^{2+}\) concentration activates a set of enzymes of the citrate cycle including pyruvate dehydrogenase, NAD\(^+\)-isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (Moreno-Sánchez and Hansford 1988; Richter and Kass 1991), which results in an increase in NAD(P)H/NAD(P)\(^+\) ratio (Duchen et al. 1993). In their reduced states, pyridine nucleotides, i.e., NADH and NADPH, exhibit an autofluorescence at a maximum wavelength of 460 nm (Aubin 1979).

In entorhinal cortex, seizure-like events (SLEs) can easily be induced by Mg\(^{2+}\)-free artificial cerebrospinal fluid (ACSF) or 4-aminopyridine (4-AP) ACSF. However, the two models differ strikingly in that SLEs of the first model regularly evolve to late recurrent discharges that are resistant to currently used anticonvulsants (Zhang et al. 1995), whereas in the second model such late activity is seen exceedingly rarely (Lopantsev and Avoli 1998; Perreault and Avoli 1991).

In the current study, we have tested the hypothesis that an energetic failure is associated with epileptiform discharges in the two different in vitro models. We have employed the NAD(P)H autofluorescence signal to monitor alteration in the temporal cortex area 3, the perirhinal cortex, the entorhinal cortex, the subiculum, the dentate gyrus, and the ventral hippocampus were prepared in a nearly horizontal plane from Wistar rats (150–200 g) after decapitation under deep ether anesthesia. The slices were stored at room temperature in oxygenated (95% O\(_2\)/5% CO\(_2\)) ACSF, which contained (in mM) 124 NaCl, 3 KCl, 1.8 MgSO\(_4\), 1.6 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose (pH 7.4). Slices were individually transferred to a homemade recording chamber and submerged in rapidly flowing ACSF. The production of spontaneous epileptiform activity under submerged conditions requires a flow rate of about 4.5 ± 0.5 (SE) ml/min and a temperature of ~36 ± 1°C.

METHODS

Subjects

The experiments were performed as described previously (Meierkord et al. 1997). Briefly, brain slices (400 μm) containing the temporal cortex area 3, the perirhinal cortex, the entorhinal cortex, the subiculum, the dentate gyrus, and the ventral hippocampus were prepared in a nearly horizontal plane from Wistar rats (150–200 g) after decapitation under deep ether anesthesia. The slices were stored at room temperature in oxygenated (95% O\(_2\)/5% CO\(_2\)) ACSF, which contained (in mM) 124 NaCl, 3 KCl, 1.8 MgSO\(_4\), 1.6 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose (pH 7.4). Slices were individually transferred to a homemade recording chamber and submerged in rapidly flowing ACSF. The production of spontaneous epileptiform activity under submerged conditions requires a flow rate of about 4.5 ± 0.5 (SE) ml/min and a temperature of ~36 ± 1°C.

Experimental method

The fluorescence measurements were carried out using a system based on an upright microscope (Axioskop; Zeiss, Jena, Germany)
with a ×10 water-immersion objective (numerical aperture 0.3; Zeiss, Jena, Germany), a xenon light source with a monochromator (Photon Technology Instruments, Wedel, Germany), and a photomultiplier (Seeofter Mees technic, Seeoeld, Germany). Autofluorescence of NAD(P)H was excited at 360 nm and measured above 400 nm using a 390 nm dichroic mirror and a 400 nm long pass filter. The autofluorescence signal measured under these conditions is derived from both mitochondrial and cytosolic NADH and NADPH, whose auto fluorescence spectra overlap. We therefore refer to NAD(P)H, indicating the signal derived from either NADH or NADPH or both. Under these conditions, an increase in auto fluorescence signal indicates an increase in the reduced state of the pyridine nucleotide, i.e., NAD(P)H, and a decrease in auto fluorescence signal an increased oxidation to NAD(P)+.

Changes in NAD(P)H auto fluorescence are given in reference to the onset NAD(P)H signal as ΔF/F₀. Except for NAD(P)H, auto fluorescence in mammalian cells is mainly influenced by the flavoprotein auto fluorescence of α-ketoglutarate and pyruvate dehydrogenase (excitation/emission maximum 450/515 nm) and the absorption by cytochrome oxidase (415–550 nm; Chance et al. 1979). However, the clearly different excitation maximum from NAD(P)H versus flavoproteins and cytochrome oxidase enables a sufficient separation of the NAD(P)H auto fluorescence signal with the use of a suitable optical filter combination (Benson et al. 1979).

Ion-selective microelectrodes were prepared using the method described by Lux and Neher (1973). Double-barreled glass was filled on one side with 154 mM NaCl operating as the reference. The silanized ion sensitive barrel (5% trimethyl-1-chlorosilane in 95% CCl₄) was filled with a potassium ionophore 1 cocktail A60031 Fluka. A modified Nernst equation was employed to obtain molar K⁺ concentrations from recorded potential values (mV).

The viability of the slices was also tested and monitored by observing the extracellular field potential in the medial entorhinal cortex layer III/IV following stimulation of the lateral entorhinal cortex. Epileptiform activity was induced by omitting Mg²⁺ from or adding 250 µM 4-AP (Sigma) to the perfusion solution. Data were stored on chart recorder or computer hard disc and subsequently analyzed off-line. All values are given as means ± SE. Statistical differences were assessed by analysis of variance (ANOVA) and Bonferroni-Dunn contrast.

RESULTS

In 19 of 19 slices spontaneous series of SLEs could be recorded in the medial entorhinal cortex. The frequency of low-Mg²⁺–induced SLEs was 0.47 ± 0.14 min⁻¹; their duration was 26 ± 1.3 s (n = 10). During SLEs, extracellular potassium concentration [K⁺]₀ increased to 6.9 ± 0.1 mM (Fig. 1A). Late, recurrent discharges (not shown) occurred 2 h (110–130 min) after the first SLE. After exposure to 250 µM 4-AP, SLEs were observed at a frequency of 0.32 ± 0.1 min⁻¹; the duration was 19 ± 1.5 s, and during SLEs [K⁺]₀ reached 7.3 ± 0.1 mM (n = 9; Fig. 1B). Late, recurrent discharges were not observed in 9 out of 9 slices after a maximal exposure time of >3 h.

The NAD(P)H auto fluorescence signal was recorded from an area of 0.8–1.5 mm² in the medial entorhinal cortex. In the absence of epileptiform activity, the NAD(P)H auto fluorescence signal was stable in both low-Mg²⁺– and 4-AP–containing perfusion solution. During SLEs the NAD(P)H auto fluorescence displayed a characteristic time course in both models: a short initial decrease was followed by a long-lasting increase (see Fig. 1). The signal decrease occurred simultaneously with the electrical onset of SLE. The duration of this NAD(P)H decrease during SLEs occurred during low-Mg²⁺–induced SLEs 2.77 ± 0.67 s and during 4-AP–induced SLEs 3.18 ± 0.51 s, and showed no significant difference between the two models (P > 0.2; Fig. 2B) and the number of SLEs. The subsequent elevation in NAD(P)H auto fluorescence developed during SLE and lasted considerably longer than the SLE. Depending on the time after onset of the first SLE, the amount of NAD(P)H increase during SLEs showed significant differences in low-Mg²⁺– and the 4-AP–containing ACSF. In Mg²⁺–free medium the SLEs induced overshoot in NAD(P)H auto fluorescence showed a reduction with time. Even after 40 repetitive 4-AP–induced SLEs the changes in NAD(P)H auto fluorescence remained unaltered (see Fig. 2A).

Moreover, the amount of NAD(P)H increase during 4-AP–induced SLE when compared with the early SLEs of the low-Mg²⁺ model was significantly smaller. In the low-Mg²⁺ model, elevation in NAD(P)H auto fluorescence declined significantly after the first 50 SLEs from 4.58 ± 0.48 to 0.79 ± 0.3. The increase in NAD(P)H signal during SLE diminished significantly after the first 4 SLE in the low-Mg²⁺ model (P < 0.05). Changes in NAD(P)H auto fluorescence signal during the first 50 4-AP–induced SLE amounted to 2.1 ± 0.08 (range 1.94–2.22). Neither the SLE-induced rises in [K⁺]₀ nor the field potentials showed significant alterations in the low-Mg²⁺ model or in the 4-AP model.

DISCUSSION

As seen in previous experiments (Meierkord et al. 1997), SLEs changed their appearance under submerged conditions in that there was a reduction in amplitude and in duration and an increase in frequency compared with interface conditions. In addition, under submerged conditions the transformation to late recurrent discharges was delayed (2 h after the first SLE), which appeared under interface conditions after 1–2 h in Mg²⁺–free ACSF (Zhang et al. 1995), respectively 50 min after the first seizure (Pfeiffer et al. 1996). It has to be emphasized that the current investigation only covers the energetic changes during recurrent SLEs.

The results of the current study demonstrate significant differences in the cellular energy metabolism associated with SLEs of the low-Mg²⁺ and the 4-AP model of epileptiform activity. The principal feature of initial brief reduction and long-lasting overshoot of the NAD(P)H signal during a single SLE was comparable in the two models. This is in line with previous studies in which, calculated from metabolites in frozen sections, a decrease was seen in the cytoplasmic-free NAD⁺/NADH ratio, during electroconvulsive seizures (Merrill and Guynn 1976), fluorothyl induced seizures (Folbergová et al. 1985), and bicuculline induced status epilepticus (Fujikawa et al. 1988). The pyridine nucleotide NAD⁺ is predominantly oxidized under normal aerobic conditions in most tissues (Thurmann and Lemasters 1988). Therefore effects, even a slight rise in NADH, caused a clear decrease in NAD⁺/NADH ratio (Merrill and Guynn 1982). In contrast to our study, these investigations only covered the time course after one event, a method that does not allow for the study of dynamic changes following series of SLEs. Furthermore, the temporal resolution in such biochemical approaches fails to monitor early changes at the onset of an
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Fig. 1. A: low-Mg$^{2+}$-induced epileptiform activity. Left: time course of changes in NAD(P)H autofluorescence (first line), extracellular K$^+$ concentration (second line), and field potentials (f.p., third line) during 4 recurrent SLEs. Right: expanded time scale for the 1st and 40th SLE. Note the decline of NAD(P)H autofluorescence overshoot, which appears already within the first 4 SLEs. B: 4-AP-induced epileptiform activity. Left: similar presentation of NAD(P)H autofluorescence (first line), extracellular K$^+$ concentration (second line), and field potentials (f.p., third line) during 4 recurrent SLEs. Right: expanded time scale demonstrates that there is no change of the NAD(P)H autofluorescence overshoot even after 40 SLEs.

SLE; this may explain why the initial brief decrease of the signal has not been demonstrated before.

The initial reduction in NAD(P)H signal represents the Ca$^{2+}$-induced depolarization of the mitochondrial membrane and therefore increased activity of mitochondrial respiratory chain (Duchen 1992). The subsequent elevation in NAD(P)H
signal indicates an activation of Ca\(^{2+}\)-dependent enzymes of the citrate cycle (Duchen et al. 1993). Accordingly, more electrons reach the mitochondrial respiratory chain via transport of NADH and were used here to drive mitochondrial proton pumps. Therefore the potential difference across the inner mitochondrial membrane increases and induces an augmentation in mitochondrial ATP production. The enlarged neuronal energy requirement during epileptic activity results in an elevated consumption of ATP (Fujikawa et al. 1988). To cover the increased use of ATP, an elevated energy production is required. Monitoring the \(\text{NAD(P)H} \) autofluorescence signal thus gives insight into the changes of intracellular energy metabolism during SLEs. Changes in energy metabolism induced by SLEs with the use of 4-AP and indicated by \(\text{NAD(P)H} \) autofluorescence signal were stable throughout the experiments (\(\approx 40\) SLE). This implies that neurons could compensate for the increased energy requirement during 4-AP-induced epileptiform activity. They may therefore only rarely display late recurrent discharges.

In contrast, a significant higher but gradual decline of the overshoot of the \(\text{NAD(P)H} \) autofluorescence signal was already observed during the first 10–15 low-Mg\(^{2+}\)-induced SLEs. After 35–40 recurrent SLEs, the overshoot had almost vanished. Our findings correspond well with hypotheses that attribute the transition into late activity in the low-Mg\(^{2+}\) model to an energetic failure. A gradual decrease afterhyperpolarization following each SLE in the low-Mg\(^{2+}\) model was demonstrated with intracellular recordings (Schmitz et al. 1997). It is suggested that this decreased hyperpolarizations could result from a rundown of the electrogenic Na\(^{+}\)-K\(^{-}\)-ATPase. The pump-induced hyperpolarizations would inactivate voltage dependent Na\(^{+}\) and Ca\(^{2+}\) inward currents and NMDA gated channels (Fukuda and Prince 1992a,b). The gradual decrease in the overshoot of the \(\text{NAD(P)H} \) autofluorescence after each SLE in the low-Mg\(^{2+}\) model could result in decreased activity of the electrogenic Na\(^{+}\)-K\(^{-}\)-ATPase following gradual reductions of intracellular ATP concentrations.

Furthermore, an observed decrease in GABA\(_{A}\) receptor-mediated inhibition in the low-Mg\(^{2+}\) model may result from a loss of ATP and a consequent partial dephosphorylation.
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of GABA$_A$ receptor (Whittington et al. 1995). The increased consumption of MgATP in the absence of intracellular Mg$^{2+}$ may result in a decrease in MgATP level and furthermore, in a loss in compensation of increased energy requirement. This group showed that the erosion of GABAergic inhibition could be reversed by the application of MgATP, but not by an increased [Mg$^{2+}$], only (Whittington et al. 1995).

Our data underline that the relative energy may contribute to the drug resistance of the late recurrent discharges in the low-Mg$^{2+}$ model and after long periods of status epilepticus in humans.

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