Mitochondrial Inhibition Prior to Oxygen-Withdrawal Facilitates the Occurrence of Hypoxia-Induced Spreading Depression in Rat Hippocampal Slices

Florian J. Gerich, Sebastian Hepp, Irmelin Probst, and Michael Müller

1Zentrum für Physiologie und Pathophysiologie, Abteilung Neuro- und Sinnesphysiologie, 2Zentrum Biochemie, and 3Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, Georg-August-Universität Göttingen, Göttingen, Germany

Submitted 28 September 2005; accepted in final form 4 April 2006

Gerich, Florian J., Sebastian Hepp, Irmelin Probst, and Michael Müller. Mitochondrial inhibition prior to oxygen-withdrawal facilitates the occurrence of hypoxia-induced spreading depression in rat hippocampal slices. J Neurophysiol 96: 492–504, 2006. First published April 12, 2006; doi:10.1152/jn.01015.2005. Oxygen withdrawal blocks mitochondrial respiration. In rat hippocampal slices, this triggers a massive depolarization of CA1 neurons and a negative shift of the extracellular DC potential, the characteristic sign of hypoxia-induced spreading depression (HSD). To unveil the contribution of mitochondria to the sensing of hypoxia and the ignition of HSD, we modified mitochondrial function. Mitochondrial uncoupling by carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 μM) prior to hypoxia hastened the onset and shortened the duration of HSD. Blocking mitochondrial ATP synthesis by oligomycin (10 μg/ml) was without effect. Inhibition of mitochondrial respiration by rotenone (20 μM), diphenylethionium (25 μM), or antimycin A (20 μM) also hastened HSD onset and shortened HSD duration. 3-nitropropionic acid (1 mM) increased HSD duration. Cyanide (100 μM) hastened HSD onset and increased HSD duration. At higher concentrations, cyanide (1 mM), azide (2 mM), and FCCP (10 μM) triggered SD episodes on their own. Compared with control HSD, the spatial extent of the intrinsic optical signals of cyanide- and azide-induced SDs was more pronounced. Monitoring NADH (nicotinamide adenine dinucleotide) and FAD (flavin adenine dinucleotide) autofluorescence and mitochondrial membrane potential verified the mitochondrial targeting by the drugs used. Except 1 mM cyanide, no treatment reduced cellular ATP levels severely and no correlation was found between ATP, NADH, or FAD levels and the time to HSD onset. Therefore ATP depletion or a cytosolic reducing shift due to NADH/FADH2 accumulation cannot serve as a general explanation for the hastening of HSD onset on mitochondrial inhibition. Additional redox couples (glutathione) or events downstream of the mitochondrial depolarization need to be considered.

Introduction

Mitochondrial dysfunction is a catastrophic event threatening cell survival. Acute or chronic mitochondrial disturbance as well as accumulating mitochondrial damage are being considered as key players in the progression of various neurodegenerative diseases such as Parkinsonism, Alzheimer’s disease, amyotrophic lateral sclerosis, and ischemic stroke (Cooper and Schapira 1997; Duchen 1999; Nicholls and Budd 2000). Several central neurons react rapidly to oxygen shortage, but the mechanisms triggering their early response and the exact involvement of mitochondria in these events are still unclear. Because mitochondria require a continuous oxygen supply, they might be the primary sensors of hypoxia, and their function might be impaired early. This is suggested by previous studies, which reported rapid depolarization of mitochondria in hypoxic/anoxic hippocampal slices (Bahar et al. 2000; Schuchmann et al. 2000). Therefore one might suspect that mitochondria act as metabolic sensors, preparing their host cells for metabolic compromise and adapting neuronal network activity to metabolic supply and demand. Such mitochondrial sensor functions have been discussed for arterial chemoreceptors (Lopez-Barneo et al. 2001; Waypa et al. 2001). Also, we earlier found evidence that mitochondria apparently initiate the anoxic response of dorsal vagal neurons (Müller et al. 2002).

The four complexes of the mitochondrial respiratory chain are at the core of cellular energy production. While transferring electrons from NADH (nicotinamide adenine dinucleotide) to oxygen, three of the four respiratory complexes (complexes I, III, and IV) extrude protons from the mitochondrial inner space (matrix) into the cytosol. The resulting inwardly directed proton gradient across the inner mitochondrial membrane—also referred to as proton motive force—gives rise to the mitochondrial membrane potential (ΔΨm), and it drives the mitochondrial ATP synthase (also termed FodF1 ATPase or complex V) (Mitchell 1961). Therefore short-circuiting the proton gradient by protonophores or inhibition of the respiratory chain, e.g., by lack of oxygen will inevitably affect mitochondrial ATP synthesis. During such mitochondrial failure, the only source available for ATP production remains glycolysis, which is usually not sufficient to maintain undisturbed cellular function. Yet ATP is not the only mitochondria-derived messenger. Mitochondrial metabolism also generates reactive oxygen species (Boveris and Chance 1973) and modulates the cytosolic redox couples NAD/NADH and FAD/FADH2 (flavin adenine dinucleotide) (Schuchmann et al. 2001; Shuttleworth et al. 2003), all of which may potentially act as cellular signaling molecules (Dröge 2002; Park et al. 1995).

To assess what extent neuronal tolerance and responses to hypoxia are modulated by mitochondrial (dys-)function, we blocked mitochondrial metabolism at various sites and probed for changes in the susceptibility of hippocampal slices to hypoxic spreading depression (HSD). HSD resembles a synchronized massive depolarization of neurons and glial cells that...
gives rise to a negative deflection of the extracellular DC potential and results in the loss of neuronal excitability (for recent reviews, see Somjen 2001, 2004). Once ignited focally, it slowly spreads out in neural tissue such as cortex (Basarsky et al. 1998), hippocampus (Aitken et al. 1998; Basarsky et al. 1998), retina (Martins-Ferreira et al. 2000), cerebellum (Nicholson 1984), and brain stem (Richter et al. 2003) at a velocity of a few millimeters per minute. Spreading depression is generated by the concerted activation of several types of cation channels acting as parallel pathways and mediating massive neuronal Na⁺ and Ca²⁺ influx (Kager et al. 2002; Müller and Somjen 1998, 2000a;b; Somjen 2001). The contribution of mitochondria to the process is not clear.

Mitochondria immediately react to anoxia (Schuchmann et al. 2000), and they depolarize strongly during spreading depression (Bahar et al. 2000), yet details of their responses and their role in signaling are poorly understood. To analyze the impact of the severity and the site of mitochondrial inhibition, we applied various inhibitors selectively targeting respiratory complexes I, II, III, or IV: rotenone, 3-nitropropionic acid (3-NPA), antimycin A, and cyanide, respectively. Alternatively, mitochondria were depolarized by the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) or mitochondrial ATP synthesis was targeted by the inhibitor of the F₄F₁ ATPase, oligomycin. Under these conditions of mitochondrial impairment, we analyzed changes in the characteristic HSD parameters and correlated them to changes in the mitochondria-controlled parameters (cellular ATP, NADH, FAD, and ΔVm) induced by the very drug treatment.

METHODS

Preparation

Hippocampal tissue slices (400 μm thickness) were prepared from ether anesthetized, 4- to 8-week-old Sprague-Dawley rats (150–300 g body wt, mostly males) as described earlier (Hepp et al. 2005; Müller and Somjen 2000a). Recordings were performed in an interface recording chamber (Oslo style) at 35–36°C which was aerated with 95% N₂-5% CO₂; flow rate 4 ml/min). Hypoxia was aeration with 95% O₂-5% CO₂ to adjust pH to 7.4. Rotenone, diphenylamine sulfoxide (DMSO) as 10–12.5 mM stocks and stored at 4°C.

Solutions

Chemicals—unless otherwise mentioned—were obtained from Sigma-Aldrich. The ACSF contained (in mM) 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 dextrose; aerated with 95% N₂-5% CO₂; flow rate 4 ml/min). Hypoxia was induced by switching the chamber’s gas supply to 95% N₂-5% CO₂; the aeration of ACSF with 95% O₂-5% CO₂ was continued during hypoxia. Reoxygenation was started 20 s after HSD onset.

Biochemical determination of cellular ATP levels

Cellular ATP levels were determined spectrophotometrically by quantifying the reduction of NADP in a coupled reaction with glucose-6-phosphate dehydrogenase (Lamprecht and Trautschold 1974; Wilken et al. 2000)

\[
\text{hexokinase} \quad \text{d-glucose + ATP} \rightarrow \text{glucose-6-P + ADP}
\]

\[
\text{Glucose-6-P-dehydrogenase} \quad \text{glucose-6-P + NADP} \rightarrow \text{gluconate-6-P + NADPH}_2
\]

Hippocampal slices were prepared as usual, the hippocampal forma-
tion was isolated and incubated in the interface chamber for 1.5 h (35.5–36°C). Slices then underwent drug treatment for 25 min while control slices remained in ACSF. Each hippocampal slice was then rapidly transferred into 300 μl of ice-cold perchloric acid (8%), immediately homogenized by 5- to 10-s sonification (Cell Disruptor W-220F, Heat Systems-Ultrasonics), and centrifuged for 10 min at 14,000 rpm (5804R, Eppendorf). The protein pellet was frozen for later Bradford determination of protein levels (Bradford 1976). The supernatant was neutralized (pH 7–8) with KHCO₃ (pure salt), centrifuged once again (Lamprecht and Trautschold 1974; Wilken et al. 2000), and frozen overnight.

The determination of ATP levels is based on the formation of NADPH₂, which was detected as increased absorption at 366 nm (1101M photometer, Eppendorf Gerätebau). The 200-μl sample volume was added to a 0.5-ml quartz cuvette and supplemented with 200 μl test solution [composition: 91.5 mM triethanolamin-hydrochlorid (AppliChem), 1 mM NADP, 100 mM D-glucose, 20 mM MgCl₂]. First, 1 μl 1:20 diluted stock of glucose-6-phosphate dehydrogenase (corresponds to 0.25 μg protein) was added to metabolize traces of glucose-6-P, and the reaction was then started by addition of 1 μl hexokinase (0.7 units) while NADPH₂ absorption was measured continuously (Fig. 7A). NADPH₂ concentrations were determined.
using an NADPH$_2$ extinction coefficient of $\varepsilon_{340}$ = 3.4 cm$^{-1}$/Mol (Lamprecht and Trautschold 1974). Due to the 1:1 stoichiometry they report directly the ATP content of the sample which was normalized to protein content (nmol ATP/µg protein).

Statistics

The data were obtained from 109 rats (92 males, 17 females), using up to five slices from each brain and performing each experiment on at least three different rats. Obvious differences in the characteristic HSD parameters in male and female rats were not observed. Data are given as means ± standard deviations. Statistical significance of the observed changes was tested by a two-tailed, unpaired Student’s $t$-test using a significance level of 5%. In the case of paired observations, a one-sample $t$-test was used to compare normalized drug effects against pretreatment control conditions. Significant changes are marked by asterisks (*$P < 0.05$, **$P < 0.01$).

RESULTS

Modulation of HSD

Under control conditions, HSD occurred within 154.1 ± 59.7 s on oxygen withdrawal. The associated extracellular DC potential shift ($\Delta V_{sc}$) had an amplitude of −16.4 ± 5.4 mV, and, measured at the half-amplitude level (Fig. 1A), a duration of 47.2 ± 11.3 s ($n$ = 158). As shown previously, HSD can be induced repeatedly in a given slice, if oxygen is readmitted in time. Such repeated hypoxia hardly affects the characteristic HSD parameters: the time to HSD onset and the DC-potential amplitude tend to decrease and HSD duration tends to increase (Müller and Somjen 1998). For the first three HSD episodes, these changes are not statistically significant (Müller and Somjen 1998). Nevertheless, to account for these tendencies, any drug-induced changes in HSD parameters were referred to a control HSD induced in each slice before drug administration and were then statistically compared with the second HSD induced in either untreated or solvent (DMSO, EtOH)-treated control slices (control series data are shown in Fig. 1B).

As working concentrations for the various drugs, we chose those used in previous studies with acute brain tissue slices: 20 µM rotenone, 20–25 µM DPI, 20 µM AMC-A, 0.1–1 mM CN, 1 mM 3-NPA, 1 µM FCCP, and 10 µg/ml oligomycin (Allen et al. 2005; Müller et al. 2002; Schuchmann et al. 2000; Wei et al. 2004). Interfaced slices are exposed to flowing bathing solution on one surface only. Therefore to ensure sufficient diffusion into the tissue, drugs were applied for 20–25 min before HSD was induced.

Uncoupling of mitochondria by FCCP (1 µM) or block of the mitochondrial F$_{0}$F$_{1}$ ATPase by oligomycin (10 µg/ml, ~12.5 µM) failed to trigger spontaneous spreading depression (SD) episodes. Inducing HSD by oxygen withdrawal during oligomycin treatment did not affect HSD at all ($n$ = 8). In contrast, FCCP hastened the onset of HSD by 31.0 ± 11.6%, reduced its amplitude by 28.3 ± 15.5%, and decreased its duration by 28.0 ± 25.0% ($n$ = 7, Fig. 1). In addition, the DC potential showed a transient positive overshoot on reoxygenation (Fig. 1, ↓). Because the F$_{0}$F$_{1}$ ATPase may reverse its direction of operation in the presence of uncouplers and hydrolyze ATP to stabilize the mitochondrial membrane potential (Duchen 1999; Nicholls and Budd 2000), we combined oligomycin and FCCP. Even on inhibition of the ATP-synthase by oligomycin, however, FCCP still hastened HSD onset by 28.0 ± 15.4% ($n$ = 7, Fig. 1). Alternatively, to account for the quite different molecular weights of oligomycin and FCCP (791 and 254 g/mol, respectively) and the likely differences in tissue penetration, we incubated slices first for 15 min with 10 µg/ml oligomycin, followed by the combined application of oligomycin and FCCP for 20–25 min. Under these conditions, the onset of HSD was still hastened by 20.9 ± 12.8%. HSD amplitude was not affected and HSD duration was slightly reduced, as previously seen with FCCP alone ($n$ = 5, Fig. 1B, data set Oligom, FCCP+Oligom).

Next we tested the effects of impaired mitochondrial respiration. The complex I blocker rotenone (20 µM) hastened HSD onset by 26.4 ± 19.3% and decreased HSD duration by 21.4 ± 18.9% ($n$ = 10; Fig. 2). DPI (25 µM), a less specific complex I blocker, also hastened HSD onset by 16.7 ± 20.0% and decreased HSD duration by 16.5 ± 18.7% ($n$ = 8). HSD amplitude was not significantly reduced by either rotenone or DPI, but both drugs induced a transient positive overshoot of the DC potential on reoxygenation (see Fig. 2A, ↓). The complex II inhibitor 3-NPA (1 mM) did not significantly affect the time to onset or the amplitude of HSD, but it increased HSD duration by 72.1 ± 66.4% ($n$ = 11, Fig. 2). Blocking complex III by antimycin A (20 µM) slightly decreased the time to HSD onset, by 17.7 ± 19.3%, and shortened HSD duration by 24.3 ± 25.8%, but did not affect HSD amplitude ($n$ = 8). Antimycin A also caused a transient positive shift of the DC potential on reoxygenation (Fig. 2A). Complex IV block by cyanide (100 µM) hastened HSD onset by 27.8 ± 10% and increased HSD duration by 26.7 ± 33.3%; HSD amplitude was not affected ($n$ = 6, Fig. 2). Combined application of rotenone and antimycin triggered spontaneous SD episodes in three of six slices tested; in the other three slices, HSD onset was hastened by 23.3 ± 9.6%, its duration was decreased by 24.8 ± 6.8%, and the amplitude was reduced by 21.6 ± 9.6% ($n$ = 3). An overview of the effects induced by the various mitochondrial blockers is presented in Table 1.

As none of the preceding treatments consistently triggered spontaneous SD episodes, some of the drugs were applied at higher concentrations. For these trials, we used the water-soluble and well-diffusible compounds CN$^{-}$ and azide as well as FCCP. Despite the presence of oxygen, 1 mM CN$^{-}$ induced a spontaneous SD episode. It occurred within 251.3 ± 34.4 s of CN$^{-}$ treatment, had an amplitude of −11.2 ± 1.1 mV, and lasted 84.2 ± 28.8 s ($n$ = 7, Fig. 3). Azide (2 mM), another complex IV inhibitor, also triggered a spontaneous SD, which occurred within 546 ± 90 s of azide treatment, had an amplitude of −17.7 ± 2.9 mV, and lasted 147 ± 57 s ($n$ = 8). These SDs induced by CN$^{-}$ and azide in the presence of oxygen clearly differed from control HSDs (Figs. 3 and 4A). They differed in SD onset time and duration and showed an incomplete recovery that presumably reflects the slow tissue penetration and wash-out of CN$^{-}$ and azide. Application of 10 µM FCCP induced a spontaneous SD episode in 12 of 14 slices. The time to SD onset was quite long, 1189 ± 342 s, whereas the duration was unusually short, only 20.1 ± 9.2 s; the amplitude of −16.9 ± 4.9 mV was comparable to HSD ($n$ = 12, Fig. 3).

Due to the pronounced differences in the electrical signs of these SD episodes, we wondered whether their spreading velocity and their invasion of the hippocampal formation also
differ from HSD. To address this question, we monitored the SD-associated intrinsic optical signal (IOS) that outlines the invaded tissue. The IOS associated with both SD and HSD is characterized by a moderate decrease in tissue reflectance shortly before HSD onset, followed by a prominent increase in tissue reflectance that coincides with the negative DC-potential deflection and the massive depolarization of neurons and glial cells (Aitken et al. 1999; Andrew et al. 1999; Kreisman et al. 2000; Müller and Somjen 1999). Having the unique advantage of being detectable by a noninvasive approach, not requiring any fluorescent markers, and yielding two-dimensional spatiotemporal information, the IOS visualizes the site of SD ignition, its spreading velocity and range. The IOS signals of $\text{K}^+$-induced normoxic SD and HSD have been shown earlier to be similar (Aitken et al. 1999), but SD induced by CN$^-$, azide, and FCCP in the presence of oxygen turned out to differ from drug-free HSD (Fig. 4).

During drug-free HSD the reflectance increased by 11.6 ± 3.3%, reached its maximum 44 s after HSD onset, and spread at a velocity of $6.5 \pm 3.2$ mm/min ($n = 14$). In the case of CN$^-$, azide-, and FCCP-evoked SD, the reflectance increase also coincided with the onset of the DC potential shift, but the
peak intensities of 14.5 ± 4.9% (n = 7), 12.9 ± 3.0% (n = 8), and 21.8 ± 7.4% (n = 9) were reached more slowly, 122, 70, and 70 s after SD onset, respectively (Fig. 4C). The highest IOS intensity was seen with the FCCP-induced SD (Fig. 4C). The IOS of the drug-induced SDs recovered more slowly and only incompletely, especially in the case of CN⁻- and azide-induced SD, as were the associated DC potential shifts (Fig. 3).

In the absence of drugs, when hypoxia was continued after HSD onset for an additional 5 min, the recovery of the IOS became similarly incomplete (data set hypoxia + 5 min plotted in Fig. 4C). As can be seen from the slice pictures in Fig. 4B which illustrate the maximal extension of IOS during various SD episodes, the hippocampal area invaded by the IOS was larger during CN⁻- and azide- than during hypoxia-induced SD, whereas it did not differ in the case of FCCP-induced SD. The IOS propagation velocity of HSD, CN⁻-, azide-, and FCCP-induced SD did not differ (Fig. 4A).

TABLE 1. Overview of effects

<table>
<thead>
<tr>
<th>Drug/Condition</th>
<th>HSD Parameters</th>
<th>Mitochondrial Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude</td>
<td>Onset</td>
</tr>
<tr>
<td>Rotenone</td>
<td>—</td>
<td>↓</td>
</tr>
<tr>
<td>Diphenyleneiodonium</td>
<td>—</td>
<td>↓</td>
</tr>
<tr>
<td>3-Nitropropionic acid</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Antimycin</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Cyanide (100 μM)</td>
<td>—</td>
<td>↓</td>
</tr>
<tr>
<td>Cyanide (1 mM)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>FCCP</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

Overview of the changes induced by the different mitochondrial inhibitors on hypoxia-induced spreading depression (HSD) parameters (amplitude, time to onset, and duration) and the investigated mitochondrial parameters (Rh123 fluorescence, NADH, and FAD autofluorescence, and ATP levels). ↑, increase; ↓, decrease; —, no change; n.d. not determined. Tendencies are indicated when the level of significance (P < 0.05) was not quite reached.

Changes in mitochondrial membrane potential (ΔΨm) and metabolism

The effects of the mitochondrial inhibitors on the characteristic HSD parameters were unexpected because some of the drugs hastened the onset of HSD, but at the same time, shortened its duration, i.e., improved the recovery from HSD, which involves normalization of membrane potentials and ion distributions and therefore requires ATP (Rosenthal and Sick 1992). Because the preparation was consistently re-oxygenated.
20 s after HSD onset, the shortening of HSD duration cannot be attributed to the earlier HSD onset. As analyzed in 130 slices, HSD duration and the time to HSD onset are not correlated (correlation coefficient = 0.049, Fig. 5). Also the molecular weight of the drugs used, and the resulting differences in tissue penetration, cannot hold as a general explanation for the different effects on the characteristic HSD parameters. Therefore to understand the drug-induced modulation of HSD, we also analyzed the effects of the various drugs on mitochondrial membrane potential (ΔΨm), mitochondrial metabolism (NADH/FAD autofluorescence) and ATP levels. An overview of all findings is presented in Table 1.

Mitochondrial membrane potential was probed using rhodamine 123 (Rh123), which reports mitochondrial depolarization by an increase in fluorescence emission (Duchen 1999; Emaus et al. 1986; Müller et al. 2005). The isolated hippocampal formation was bulk loaded in 4–5 g/ml Rh123 for 25–30 min, and the changes in Rh123 fluorescence resulting from mitochondrial inhibition were quantified for a region of interest in st. radiatum of the CA1 region (see Fig. 6A for summary). These experiments were performed in submerged slices (34°C) to improve the detection efficiency of fluorescence emission. Due to the faster drug-penetration in submerged slices, drugs were applied for 15 min; CN− acted rapidly and was applied...
for 5 min only. A complete recovery of Rh123 levels on washout could only be observed for CN− (Fig. 6A) because most drug effects were only poorly reversible or Rh123 levels even kept increasing very slowly after drug withdrawal (rotenone and AMC-A, see Fig. 6A). A clear depolarization of mitochondria was induced by rotenone (22.8 ± 8.2%, n = 5), DPI (35.7 ± 7.9%, n = 5), antimycin A (15.4 ± 5.4%, n = 5), CN− (1 mM 34.3 ± 11.3% n = 5; 100 μM 12.2 ± 9.0% n = 6), and FCCP (35.3 ± 14.7%, n = 6). In contrast, 3-NPA and oligomycin caused only moderate changes in mitochondrial membrane potential (4.8 ± 2.0%, n = 6 and −2.6 ± 1.9%, n = 7, respectively, Fig. 6A).

To quantify the drug-induced effects on mitochondrial metabolism, we measured changes in NADH and FAD autofluorescence, which are a direct measure for mitochondrial metabolism and redox state (Chance and Williams 1955; Mills and Jobis 1972; Shuttleworth et al. 2003) (Fig. 6B). Experiments were performed similarly to the Rh123 recordings—except...
that the recording of autofluorescence does not require dye loading. The two compounds contributing to cellular autofluorescence are reduced NADH and oxidized FAD. Therefore changes in mitochondrial substrate utilization cause opposite changes in these two measures. An increase in NADH levels and the corresponding decrease in FAD levels occurred in response to rotenone and CN⁻, indicating inhibition of mitochondrial respiration (Fig. 6B); antimycin A clearly increased NADH levels but hardly affected FAD. As expected from mitochondrial uncoupling, FCCP decreased NADH and increased FAD levels, indicating stimulation of mitochondrial respiration. Against expectation, DPI also decreased NADH and increased FAD levels. Unclear is, why oligomycin slightly decreased NADH and increased FAD levels (Fig. 6B).

Changes in cellular ATP levels

Cellular ATP levels may affect the time to HSD onset (Allen et al. 2005; Roberts and Sick 1992) and depletion of ATP—as mimicked by pharmacological inhibition of Na⁺/K⁺-ATPase—triggers SD despite the presence of oxygen (Balestrino et al. 1999). We found HSD onset to be hastened by ATPase—triggers SD despite the presence of oxygen (Balestrino et al. 1999). We found HSD onset to be hastened by ATPase—triggers SD despite the presence of oxygen (Balestrino et al. 1999). We found HSD onset to be hastened by ATPase—triggers SD despite the presence of oxygen (Balestrino et al. 1999). We found HSD onset to be hastened by ATPase—triggers SD despite the presence of oxygen (Balestrino et al. 1999).

To decide whether our observed changes in HSD parameters—especially the hastened onset—could be a consequence of a severe reduction in ATP levels or even ATP depletion, we compared the ATP levels in slices kept in control solution (ACSF) with those slices exposed to the various mitochondrial inhibitors (Fig. 7). The cellular ATP level of untreated slices averaged $11.4 \pm 3.8$ nmol ATP/mg protein ($n = 22$), which corresponds to the values determined by others for hippocampal slices of adult rats by using the luciferin/luciferase bioluminescence assay (Galeffi et al. 2000) ($13.3 \pm 1.1$ nmol ATP/mg protein) or HPLC (Paschen and Djuricic 1995; Riepe et al. 1997) ($13.2 \pm 1.1$ and 9.4 ± 0.5 nmol/mg protein, respectively). The various mitochondrial drugs had only moderate effects on cellular ATP levels, and most importantly only the high concentration of 1 mM CN⁻-depleted cellular ATP. Compared with control slices, 20–25 min pretreatment of slices with rotenone (20 μM), or oligomycin pretreatment (15 min, 10 μg/ml) followed by combined application of oligomycin and FCCP (1 μM) significantly reduced cellular ATP levels to 6.4 ± 1.9 nmol/mg protein ($n = 8$) and 8.1 ± 1.6 nmol/mg protein ($n = 6$), which correspond to 56.1 and 71.1% of control ATP levels, respectively (Fig. 7B). Antimycin A (20 μM), FCCP (1 μM) or oligomycin (10 μg/ml) just showed a tendency to reduce ATP levels, which did however not reach statistical significance. DPI (25 μM), 3-NPA (1 mM), and CN⁻ (100 μM) did not noticeably affect ATP levels (Fig. 7B).

Cellular ATP was completely depleted, however, when slices were exposed to 1 mM CN⁻ for 20–25 min. This was very probably the result of SD, which is induced within 4–5 min by this concentration of CN⁻ (Fig. 3).

**DISCUSSION**

Mitochondrial inhibition prior to oxygen withdrawal hastened HSD onset, i.e., increased the susceptibility of hippocam-
pal slices to hypoxia. However, not each site of mitochondrial inhibition was equally effective. Applied at higher concentrations CN\(^{-}\) (1 mM) and FCCP (10 \(\mu\)M) as well as azide (2 mM) induced spontaneous SD episodes. Monitoring mitochondrial metabolism (NADH/FAD autofluorescence), mitochondrial membrane potential (Rh123 fluorescence), and cellular ATP levels verified the modulation of mitochondrial function by the inhibitors used but also exclude ATP depletion as a general cause for the accelerated HSD onset (see Table 1 for overview). None of the respiratory chain blockers markedly decreased the amplitude of HSD. This suggests that drug treatment did not reduce the viability of slices nor did it reduce the synchronization of the massive neuronal depolarization that is required for the ignition and propagation of HSD.

Mitochondrial impairment does not necessarily decrease cellular ATP levels

Inhibition of any one of the respiratory complexes affects electron transfer within the entire mitochondrial respiratory chain (Berg et al. 2002; Nicholls and Budd 2000) and therefore may potentially interfere with mitochondrial metabolism and ATP production. Similarly, mitochondrial uncoupling by FCCP rapidly depolarizes mitochondria and potentially interferes with mitochondrial ATP synthesis that is driven by the proton gradient (proton motive force) across the inner mitochondrial membrane (Mitchell 1961). Inhibition of the ATP synthase by oligomycin directly targets mitochondrial ATP production yet without affecting the function of the respiratory chain. Although inhibition of the proximal parts of the respiratory chain (complexes I and II) can at least partly be compensated for, inhibition of the distal parts (complexes III and IV) cannot. Inhibition of complex I by, e.g., rotenone or DPI, still does allow FADH\(_2\) to be utilized and its electrons to be shuttled into the respiratory chain via complex II (Berg et al. 2002). Inhibition of succinate dehydrogenase in complex II by 3-NPA prevents the utilization of FADH\(_2\) but does not interfere with the utilization of NADH and the resulting flow of electrons from complex I to complex III via complex II (Berg et al. 2002). This may explain why 3-NPA failed to hasten HSD onset and to markedly affect mitochondrial membrane potential, NADH/FAD levels, or cellular ATP content. The prolonged HSD duration in the presence of 3-NPA suggests that the recovery after reoxygenation was delayed in the presence of 3-NPA, but the underlying mechanisms are not clear. A desynchronization of the anoic depolarization in single neurons is unlikely because this should also have reduced the HSD amplitude.

HSD onset was hastened only by those blockers that directly short-circuit the proton gradient (FCCP) or target the proton-pumping complexes I, III, and IV (rotenone, antimycin A, and CN\(^{-}\)). Therefore one might suspect that the hastened HSD onset reflects accelerated ATP depletion because cellular ATP levels, determined by glucose content and the efficiency of anaerobic glycolysis, do affect the time to HSD onset (Allen et al. 2005; Roberts and Sick 1992). Of course ATP depletion will affect the time to HSD onset but did not affect ATP levels at all (Fig. 7B). Application of 100 \(\mu\)M CN\(^{-}\) caused the most pronounced hastening of HSD onset but did not affect ATP levels at all (Fig. 7B). In contrast, oligomycin showed a clear tendency to reduce cellular ATP content (71.9% of control levels) but did not affect HSD. Our ATP measurements report the ATP level at the time point where hypoxia would have been induced; ATP levels during hypoxia before HSD onset were not measured, and due to the short time to HSD onset—on average 2.7 min of hypoxia—this had been difficult to realize. Because the entire hippocampal slice was used for ATP determination and the different hippocampal subfields may have been affected by the drugs to a different degree, the decrease in ATP levels might have been underestimated. Yet in this case, the true reduction of ATP levels by oligomycin would have been even more pronounced, but still oligomycin did not affect HSD onset. The fact is that, for the various drugs tested, a consistent correlation of hastened HSD onset and cellular ATP levels was not observed.

Further arguments against ATP depletion as a general cause for the hastening of HSD onset is the observation that FCCP, rotenone, DPI, and antimycin A also shortened the duration of HSD, i.e., they accelerated the ATP-demanding recovery of CA1 neurons and the normalization of ion levels (Rosenthal and Sick 1992). If cellular ATP had been depleted, one would have expected a slow and incomplete recovery, similar to what was observed in the case of CN\(^{-}\) and azide-induced SDs or the continuation of hypoxia after HSD onset (Figs. 3 and 4C). Furthermore, the hastening of HSD onset by FCCP persisted in the presence of oligomycin—even when oligomycin treatment was started 15 min ahead of FCCP application—indicating that accelerated ATP depletion due to mitochondrial ATP consumption by reversed F\(_{0}\)F\(_{1}\)-ATPase (Duchen 1999; Nicholls and Budd 2000) cannot be its cause. Neither did the ATP levels markedly differ when FCCP and oligomycin were applied either alone or in combination (Fig. 7B). We therefore conclude that—rather than cellular ATP levels—other factors arising from mitochondrial depolarization/dysfunction seem to be involved in the facilitated generation of HSD.

The assay of ATP levels does not differentiate between mitochondrially and glycolytically derived ATP. Therefore it is possible that the impaired mitochondrial ATP production was in part compensated by increased glycolytic ATP production (Nicholls and Budd 2000). Such a compensation as well as the additional utilization of glycogen stores and phosphocreatine have recently been demonstrated for the CA1 region of rat hippocampal slices (Allen et al. 2005). These experiments of
Allen and coworkers differ from those reported here, as juvenile rats, submerged slices, and a somewhat lower temperature (33–34°C) were used. Besides, oxygen-glucose deprivation (ischemia-like condition) was necessary to induce the anoxic depolarization (i.e., HSD). Due to these differences and the obviously lower metabolic demand of their slices, mitochondrial inhibition by hypoxia or 1 mM CN− was not sufficient to induce an SD in their experiments.

**Changes in cytosolic redox state**

Inhibiting mitochondrial respiration modulates cytosolic redox state by releasing reactive oxygen species (ROS) (Bindokas et al. 1996; Boveris and Chance 1973; Nicholls and Budd 2000) and by increasing cellular NADH levels (Foster et al. 2005; Mills and Jöbsis 1972; Schuchmann et al. 2001). Mitochondrial uncoupling by, e.g., FCCP activates mitochondrial respiration maximally (Duchen 1999), thereby decreasing NADH levels (Rex et al. 1999) and increasing ROS production (Bindokas et al. 1996). Accordingly, FCCP causes an oxidative shift in cytosolic redox state. Mimicking such oxidizing conditions by application of H2O2 or the sulfhydryl oxidizing agent DTNB (dithionitrobenzoic acid), we recently reported HSD onset to be postponed due to activation of BK channels (Hepp et al. 2005). In contrast, the intrinsic formation of ROS early during hypoxia is apparently not a major contributor to the ignition of HSD. Combined application of the radical scavengers ascorbic acid (1 mM) and trolox (0.75 mM) did not affect HSD at all (n = 9) (unpublished data F. J. Gerich and M. Müller). In support of our data, others also reported that the time to onset of the ischemia-induced anoxic depolarization in hippocampal CA1 neurons is not affected by the radical scavenger Mn(III)tetrakis(4-benzoic acid)porphyrin (MnTAP) (Allen et al. 2005). However, we found reducing conditions as mimicked by DTT (dithiothreitol) to favor the onset of HSD (Hepp et al. 2005), yet the very molecular targets involved still need to be identified. Therefore the reducing shift resulting from the inhibition of the respiratory chain might be considered a putative intracellular signal, mediated by, e.g., increased NADH, FADH2, and reduced glutathione levels.

Both anoxia and the induction of HSD (as well as normoxic SD) modify cellular NADH levels. Imaging tissue NADH changes during normoxic SD in gerbil cortex in vivo, Hashimoto and coworkers (Hashimoto et al. 2000) observed a biphasic NADH signal, consisting of an initial increase followed by a temporary decrease below baseline levels. Although the initial NADH increase could be verified biochemically, the secondary decrease could not, and they concluded that it rather reflects changes in cerebral blood flow that interfere with the detection of NADH autofluorescence (Hashimoto et al. 2000). However, when correcting for such changes in blood flow (by monitoring changes in tissue reflectance), an undershoot of the NADH baseline—referred to as mitochondrial hyperoxidation—is still found during electrical stimulation, normoxic spreading depression as well as seizures in in vivo cat cortex (Rosenthal and Somjen 1973). It therefore seems that besides a possible interference with hemoglobin-content and -oxygenation, changes in tissue oxygenation levels as a result of increased cerebral blood flow and altered O2 consumption contribute to the hyperoxidation of NADH (Rosenthal et al. 1995). Also, intracellular derangement disturbing mitochondrial metabolism possibly with a contribution of reactive oxygen species have been discussed (Perez-Pinzon et al. 1997; Rosenthal et al. 1995). By contrast, inducing HSD in vitro (acute hippocampal slices) causes a clear NADH increase already before HSD onset, and NADH reduction then becomes maximal as soon as HSD is triggered (Foster et al. 2005). Hyperoxidation of NADH does not occur unless anoxia is continued for several minutes after HSD onset. Under these conditions, tissue pO2 levels rise as a result of irreversible neuronal injury and the associated decreased oxygen consumption (Foster et al. 2005).

A clear correlation of HSD onset and reducing conditions, i.e., NADH/FAD levels, was not found in our experiments (see Table 1 for overview). Although HSD onset was hastened by FCCP, rotenone, DPI, antimycin, and CN−, a clear reducing shift, i.e., increase in NADH levels and/or decrease in FAD levels, was only induced by rotenone, antimycin A, and CN− (Fig. 6B). In contrast, FCCP and DPI decreased NADH and increased FAD levels, which should rather shift cytosolic redox balance to oxidizing conditions. However, we evaluated only two of the major redox couples within the cytosol. Glutathione, which may also modify sulfhydryl residues of redox-sensitive proteins (Dröge 2002; Lipton et al. 2002), has not been quantified so far. Due to the interaction of all these redox couples, using a general marker of cytosolic redox state would also be advantageous. Such markers have recently developed in the form of redox-sensitive fluorescent proteins (Hanson et al. 2004; Østergaard et al. 2001), and they even allow for the continuous and dynamic detection of cytosolic redox changes, but they are not yet commercially available.

A clear correlation was found, however, between mitochondrial membrane potential and the time to HSD onset. Each drug that hastened HSD onset (rotenone, DPI, antimycin A, CN−, and FCCP) also caused a clear depolarization of mitochondria (Fig. 6A). Could it be the depolarization of mitochondria that facilitates the triggering of HSD onset? But how can the mitochondrial depolarization be transmitted to the plasma-membrane channels, and what messengers might be involved? Among others, mitochondrial depolarization is expected to reduce the Ca2+ sequestration by mitochondria (Nicholls 1978; von Lewinski and Keller 2005), and it may modulate cytosolic pH (Kaila et al. 1989).

DPI hastened HSD onset, just as the complex I blocker rotenone, but otherwise it behaved quite differently. ATP levels were not affected by DPI and the observed decrease in NADH and the increase in FAD levels do not match the DPI-mediated mitochondrial depolarization. The NADH/FAD response rather indicates a stimulation of mitochondrial respiration, possibly in response to the DPI-mediated mitochondrial depolarization. Because DPI does not only target complex I but also blocks various oxidases such as NADH- and xanthine oxidase as well as NO synthase (Li and Trush 1998; Stuehr et al. 1991), additional, mitochondria-independent mechanisms might have contributed.

**Intrinsic optical signals**

The intrinsic optical signals of the hypoxia- and drug-induced SD episodes were identical in terms of the direction of the reflectance changes, yet their intensities and time course differed (Fig. 4). The very source of the scattering increase is
still unclear. According to our earlier studies, it depends on the occurrence of the electrical signs of HSD (Müller and Somjen 1998), is Cl⁻–dependent and sensitive to anion transport blockers. Nevertheless, cell swelling had to be excluded as a major source for the reflectance increase; possibly changes in cytoarchitecture such as swelling of mitochondria and other organelles might be involved (Aitken et al. 1999; Bahar et al. 2000; Müller and Somjen 1999). Because the most pronounced scattering increase occurs in the dendritic layers, others have suggested microstructural damage (“beading”) of dendrites (Andrew et al. 1999). Such changes are, however, irreversible, and their occurrence may be mostly related to ischemic conditions or maintained hypoxia. Also the curvature of the surface of interfaced slices has been suggested to contribute (Kreisman et al. 1995). Fluoroacetate poisoning of glial cells increases the intensity of the IOS during HSD, suggesting that the generation of the scattering increase does not rely on viable glial cells (Müller and Somjen 1999). In contrast, glial poisoning by fluoroacetate or mitochondrial inhibition by rotenone were found to decrease the characteristic IOS—a scattering decrease—associated with evoked neuronal activity in hippocampal slices (Buchheim et al. 2005), once more pointing out the different nature of intrinsic optical signals associated with neuronal activity and HSD.

We have now demonstrated that the intrinsic optical changes during HSD persist in the presence of drugs targeting mitochondrial metabolism and membrane potential (FCCP, CN⁻, azide); this indicates that severe mitochondrial depolarization and/or failure of mitochondrial metabolism can be excluded as a major source for the generation of the IOS associated with HSD. To what degree mitochondrial swelling is affected by the tested drugs cannot be decided at present.

Concluding remarks

In conclusion, a clear correlation exists for the depolarization of mitochondria and the hastening of HSD onset, proving a crucial role of mitochondria during the early phase of hypoxia and the determination of HSD onset. The cellular levels of ATP, NADH, and FAD did not show a clear correlation with the drug-induced changes in HSD. Therefore neither ATP depletion nor a reducing shift due to NADH, FAD accumulation can serve as a general explanation for the hastening of HSD onset in response to mitochondrial inhibition. Whether these changes may contribute in the one or other case has to be clarified in further experiments at the single-cell level. In view of the central role of mitochondrial membrane potential, altered intracellular Ca²⁺ levels as a consequence of disturbed mitochondrial Ca²⁺ sequestration (Nicholls 1978; von Lewinski and Keller 2005), Ca²⁺ release from malfunctioining mitochondria (Schuchmann et al. 2000), and subtle changes in cytosolic pH due to the depolarizing mitochondria (Kaila et al. 1989) have to be considered as putative signaling mechanisms. Unveiling the very mechanisms involved will require further detailed investigations quantifying and correlating the early changes in mitochondrial membrane potential and its effects on other cytosolic redox couples such as reduced/oxidized glutathione.

ACKNOWLEDGMENTS

We are grateful to Prof. G. G. Somjen for a critical reading of the manuscript, and we thank B. Hildebrandt for excellent technical assistance.

GRANTS

This study was supported by Deutsche Forschungsgemeinschaft Grants SFB 406, TP C14 and CMPB and by equipment grants from the Göttingen University (Ausstattungsmittel Juniorsprofessor).

REFERENCES


Ischemic cell death in brain neurons.

Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, Li PA and Siesjo BK

Mueller M and Somjen GG.

Coupling of phosphorylation to electron and hydrogen transfer by Mitchell P.

Light

Regional differences in hypoxic Fall in intracellular pH and increase in

Report

Cellular mechanism of Lopez-Barneo J, Pardal R, and Ortega-Saenz P.

Nicholson C.

The regulation of extramitochondrial free calcium ion concen-

Mueller M, Mironov SL, Ivannikov MV, Schmidt J, and Richter DW.


Wilken B, Ramirez JM, Probst I, Richter DW, and Hanefeld F. Anoxic ATP depletion in neonatal mouse brainstem is prevented by creatine supple-


Paschen W and Djuricic B. Comparison of in vitro ischemia-induced distur-


Perez-Pinzon MA, Mumford PL, Rosenthal M, and Sick TJ. Antioxidants, mitochondrial hyperoxidation and electrical recovery after anoxia in hip-


Rex A, Pfeffer L, Fink F, and Fink H. Cortical NADH during pharmaco-


Rosenthal M, Feng ZG, Raffin CN, Harrison M, and Sick TJ. Mitochon-


