Intrinsic Optical Signals in Rat Hippocampal Slices During Hypoxia-Induced Spreading Depression-Like Depolarization

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Müller, Michael and George G. Somjen. Intrinsic optical signals in rat hippocampal slices during hypoxia-induced spreading depression-like depolarization. J. Neurophysiol. 82: 1818–1831, 1999. In interfaced rat hippocampal slices spreading depression (SD) and hypoxia-induced SD-like depolarization are associated with increased light reflectance and decreased light transmittance, indicating increased light scattering. By contrast, mild hypotonicity or electrical stimulation decrease light scattering, which is usually taken to be caused by cell swelling. This difference has been attributed to experimental conditions, but in our laboratory moderate osmotic challenge and SD produced opposite intrinsic optical signals (IOSs) in the same slice under identical conditions. To decide whether the SD-induced IOS is related to cell swelling, we investigated the effects of Cl⁻ transport inhibitors and Cl⁻ withdrawal on both light reflectance and transmittance, as well as on changes in interstitial volume and tissue electrical resistance. In normal [Cl⁻], early during hypoxia, there was a slight decrease in light reflectance paired with increase in transmittance. At the onset of hypoxic SD, coincident with the onset of cell swelling (restriction of TMA⁺ space), the IOS signals suddenly inverted, indicating sharply increased scattering. The SD-related IOSs started in a single spot and spread out over the entire CA1 region without invading CA3. Application of 2 mM furosemide decreased IOS intensity. When [Cl⁻] was substituted by methylsulfate or gluconate, the SD-related reflectance increase and transmittance decrease were suppressed and replaced by opposite signals, indicating scattering decrease. Yet Cl⁻ withdrawal did not prevent cell swelling measured as shrinkage of TMA⁺ space. The SD-related increase of tissue electrical resistance was reduced when bath Cl⁻ was replaced by methylsulfate and almost eliminated when replaced by gluconate. The TMA⁺ signal is judged to be a more reliable indicator of interstitial space than tissue resistance. Neither application of cyclosporin A nor raising [Mg²⁺], depressed the SD-related reflectance increase, suggesting that Cl⁻ flux through mitochondrial “megachannels” may not be a major factor in its generation. Fluoroacetate poisoning of glial cells (5 mM) accelerated SD onset and enhanced the SD-induced reflectance increase threefold. This suggests, first, that glial cells normally moderate the SD process and, second, that neurons are the predominant generators of the light-scattering increase. We conclude that light scattering by cerebral tissue can be changed by at least two different physical processes. Cell swelling decreases light scattering, whereas a second process increases scattering. During hypoxic SD the scattering increase masks the swelling-induced scattering decrease, but the latter is revealed when Cl⁻ is removed. The scattering increase is Cl⁻ dependent, nevertheless it is apparently not related to cell volume changes. Its underlying mechanism is as yet not clear; possible factors are discussed.

INTRODUCTION

Intrinsic optical signals (IOS) of brain tissue slices have been found useful in visualizing neuronal excitation without using fluorescent dyes. They are closely associated with neuronal stimulation, excitotoxicity, epileptiform activity, cell swelling, as well as spreading depression (SD) and hypoxic SD-like depolarization, and, depending on the experimental conditions, they show marked differences with respect to their intensity, time course, and direction (Aitken et al. 1998; Basar- sky et al. 1998; Holthoff and Witte 1996; MacVicar and Hochman 1991; Meierkord et al. 1997). IOSs have usually been attributed to changes in light scattering by the tissue, and they have been taken to be a measure of average cell volume (e.g., Lipton 1973; Ørskov 1935; reviewed by Aitken et al. 1999). Besides cell volume changes, there appear, however, to be additional mechanisms that also may contribute to the generation of IOSs (Kreisman et al. 1995; Meierkord et al. 1997). The present study therefore focuses on the mechanism of IOSs associated with hypoxic SD and investigates their correlation to cell swelling.

The scattering of light influences the translucence of an object and the amount of light reflected from its surface in opposite sense: increased scattering decreases light transmittance but increases light reflectance, whereas decreased scattering causes reciprocally opposite changes. By contrast, altered absorption of light affects transmittance and reflectance in the same direction, for if light energy is converted either into heat or during a photochemical process, both reflected and transmitted light will decrease. Also, changes in the geometry of an interfaced tissue slice would alter reflected and transmitted IOS in the same sense. Reducing the radius of curvature of the surface causes the slice to resemble a convex lens, changing the angles of both reflection and refraction so as to divert light away from the detector looking down on the slice, no matter whether the light came through the tissue, or was incident on it (Aitken et al. 1999; Born and Wolf 1970; Kreisman et al. 1995).

Previous reports show that mild to moderate hypotonicity reduces the intensity of light reflected from the surface of a tissue slice while increasing the light transmitted through the tissue (Andrew and MacVicar 1994; Lipton 1973). This is in line with the expected reduction in light scattering due to hypotonic cell swelling. Electrical stimulation of cerebral tissue also causes reduced scattering in the excited region, and the resulting IOSs are diminished by application of furosemide or Cl⁻ withdrawal, confirming that they are related to cell swelling (Andrew and MacVicar 1994; Holthoff and Witte 1996; Lipton 1973; MacVicar and Hochman 1991). In contrast, during SD, a process known to be associated with marked cell swelling (Hansen and Olsen 1980; Jing et al. 1994), light scattering increases, and this change is much greater than the
scattering decrease seen under milder challenges (Aitken et al. 1998; Martins-Ferreira and Oliveira Castro 1966; Müller and Somjen 1998c; Snow et al. 1983).

In an attempt to resolve this seeming paradox, Kreisman et al. (1995) reported that light transmittance changes induced by osmolarity invert sign when the bath level is lowered so that the previously submerged slice comes to lie at the interface between liquid and gas. Kreisman et al. (1995) concluded that in submerged slices changes in scattering within the tissue generate the IOS, whereas in slices lying at a gas-liquid interface the refraction at the slice surface determines the signal. Hypotonic swelling causes the slice to bulge, i.e., it shortens the radius of curvature, resulting in less light being collected by the detector. When the slice is submerged, this surface-optical effect is abolished because the refractive indices of the bath and the tissue are similar. Kreisman et al. (1995) also pointed out that the SD-related increase of scattering has been observed in interfaced slices, whereas the swelling-related decrease was reported for submerged tissues. In keeping with these arguments, Basarsky et al. (1998) observed increased light transmittance (decreased scattering) during SD in submerged hippocampal slices. It should be remembered, however, that the isolated retina preparation produces a marked increase of light scattering during SD, which is easily seen with the naked eye, even though it is completely submerged in bathing fluid (Martins-Ferreira and Oliveira Castro 1966).

The strikingly large and sudden optical changes during hypoxic SD in hippocampal slices begin at the same time as the sudden intra- and extracellular potential shifts signaling SD onset. In an earlier study we monitored light reflectance of hippocampal slices under a variety of manipulations designed to influence SD-related biophysical changes. We found that pharmacological inhibition of hypoxic SD by blockade of the major Na\(^+\) and Ca\(^{2+}\) pathways also prevents the hypoxia-induced optical changes (Müller and Somjen 1998c). The simultaneous onset of the electrical and optical signals as well as the cell swelling during normoxic and hypoxic SD (Jing et al. 1994) seemed to reinforce the earlier assumption that the IOS reflects cell swelling, even if the scattering increase was opposite to the scattering decrease associated with moderate hypotonicity (Snow et al. 1983; Turner et al. 1995).

In the experiments presented here, we examined the relationship of the optical signals to Cl\(^-\) fluxes and to cell volume changes detected by the indicator-dilution technique (Hansen and Olsen 1980; Phillips and Nicholson 1979) as well as tissue resistance measurements (Freygang and Landau 1955). Contrary to expectation, we found that the hypoxic SD-induced light-scattering increase is not related to cell swelling or the resulting restriction in extracellular space, even though it is dependent on Cl\(^-\) flux. Metabolic poisoning of glial cells accelerated the onset of hypoxic SD and intensified the associated optical signals, indicating that viable glial cells are not required for the generation of the optical signal. Positive evidence for a major contribution of mitochondrial swelling to the generation of the optical signal was not obtained.

Parts of this study have been published in abstract form (Müller and Somjen 1998a,b, 1999; Somjen and Müller 1999).

**METHODS**

**Preparation**

Hippocampal tissue slices were prepared from male Sprague-Dawley rats (95–210 g body wt; 4–6 wk old). The rats were decapitated under ether anesthesia, and the brain was rapidly removed from the skull and placed in chilled artificial cerebrospinal fluid (ACSF) for 1–2 min. One hippocampus was isolated, and transverse slices of 400-μm thickness were cut using a tissue chopper. Slices were transferred to an interface recording chamber and were left undisturbed for 90 min. The recording chamber was kept at a temperature of 34.5–35.5°C. It was continuously aerated with 95% O\(_2\)-5% CO\(_2\) (400 ml/min) and perfused with oxygenated ACSF (1.5 ml/min). Hypoxia was induced by switching the chamber’s gas supply to 95% N\(_2\)-5% CO\(_2\). To protect the slices from drying out and to prevent oxygenation from the air during hypoxic episodes, the slice chamber was covered by a lid with a small (2 cm\(^2\)) opening for the positioning of the electrodes. Exchange of the bathing solution and diffusion of applied drugs into the slice took ~15 min.

**Solutions**

The ACSF had the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH\(_2\)PO\(_4\), 24 NaHCO\(_3\), 1.2 CaCl\(_2\), 1.2 MgSO\(_4\), and 10 dextrose; aerated with 95% O\(_2\)-5% CO\(_2\) to adjust pH to 7.4. When TMA\(^+\)-sensitive microelectrodes were used to monitor changes in extracellular space, 1.5 TMA\(^+\) (tetramethylammonium-chloride, ICN) was added. In low Cl\(^-\) solution either methylsulfate or gluconate replaced all but 3.9 mM Cl\(^-\) (as CaCl\(_2\) and TMA-Cl). Furosemide (Sigma), 4,4’-dinitrostilbene-2,2’-disulfonic acid (DNDS; TCI), and fluoroacetate (Sigma) were directly dissolved in ACSF. 4,4’-Disothiocyanostilbene-2,2’-disulfonic acid (DIDS; Sigma) was dissolved in distilled water before being added to ACSF. Glibenclamide (RBI) was dissolved in dimethyl sulfoxide (DMSO; Sigma) to prepare a 100-mM stock solution (stored at ~20°C), and diazoxide (RBI) was dissolved in 0.1 M NaOH (20 mM stock solution; ~20°C). Cyclosporin A (Calbiochem) was dissolved in absolute ethanol (25 mM stock solution; ~20°C). Immediately before the experiment, 0.1 ml DMSO were added to 0.1 ml cyclosporin A stock solution, and this mixture was then added to well-oxygenated ACSF. Final ethanol and DMSO concentrations were 0.02% for cyclosporin A solutions and 0.1% DMSO for glibenclamide solutions.

**Microelectrodes**

Single-barreled glass microelectrodes for extracellular recordings were pulled from thin-walled borosilicate glass (TW150F-4, WPI) using a horizontal puller (Flaming Brown, P-80/PC). They were filled with ACSF, and their tips were broken to a final resistance of 5–10 MΩ. Changes in extracellular space were quantified by measuring changes in the background concentration of the membrane-impermeable TMA\(^+\) ion (indicator-dilution technique) (see also Hansen and Olsen 1980; Nicholson and Phillips 1981; Phillips and Nicholson 1979). TMA\(^+\)-sensitive microelectrodes were made from double-barreled theta type capillaries (GCT 200–10, Clark Electromedical Instruments). The ion-sensitive barrel was silanized by 60-min exposure to hexamethyldisilazane vapors (HMDS 98%, Fluka; vaporized at 40°C). Silanization of the reference barrel was prevented by perfusion with compressed air (1.5 bar). The tip of the ion-sensitive barrel was filled with the Corning 477317 K\(^+\) ion exchanger (IE190, WPI) and backfilled with 150 mM TMA-Cl + 10 mM N-(2-hydroxyethyl) piperazine-N’-(2-ethanesulfonic acid) (HEPES; Sigma), pH 7.4. The reference barrel contained 150 mM NaCl + 10 mM HEPES, pH 7.4. Mean electrode resistances of the reference and ion-sensitive barrel were 20–40 MΩ and 80–110 MΩ, respectively.
solutions (0, 0.1, 0.5, 1, 5, 10, 50, and 100 mM). The ionic strength of the calibration solutions was held constant and corresponded to extracellular conditions. The electrodes did not respond to replacing O₂ by N₂, and because the backfill solution of the ion-selective barrel did not contain K⁺, [K⁺], changes had little effect on electrode potential. In the presence of 1 mM TMA⁺, increasing [K⁺], from 3.5 to 50 mM induced an electrode response of only 0.3 ± 1.3 mV. Average slope of the electrodes was 60.6 ± 2.7 mV/decade TMA⁺, and their detection limits were 0.13 ± 0.10 mM TMA⁺ (mean ± standard deviation, n = 23).

**Electrical recordings**

Electrode signals were referred to an Ag/AgCl reference electrode embedded in 2% agar in 3 M KCl. They were recorded by a DC amplifier (constructed locally) and digitized by a TL-1/Lab Master acquisition system at sampling rates of 10 Hz using the Axotape V2 software (Axon Instruments). Because electrodes were calibrated to TMA⁺ concentrations and the TMA⁺ activity coefficient was held constant, changes in [TMA⁺], could directly be calculated from the electrode responses using the electrodes’ averaged slope of pre- and postexperiment calibration. Changes in relative interstitial volume (ISV) during hypoxia were calculated as a percent of “control” according to the formula (Dietzel et al. 1980)

$$\Delta ISV(%) = 100\% \left[ \frac{[TMA^+]_{control} - [TMA^+]_{hypoxia}}{[TMA^+]_{hypoxia}} \right]$$

All signal amplitudes were measured between the prehypoxia baseline and the maximal change. The duration of hypoxic SD-associated negative DC potential deflection (ΔVₜ), IOS, and cell swelling was determined at the half-amplitude level. SD onset was defined as occurrence of the sudden (ΔVₜ).

Changes in electrical tissue resistance during hypoxia were measured using the “four-electrode” method (Freygang and Landau 1955; Li et al. 1968; Traynelis and Dingledine 1989). Two monopolar microwire stimulation electrodes, representing anode and cathode, were used to deliver constant current pulses of 3–5 μA amplitude and 20–30 ms duration every 10 s; the same pulse was used throughout any one experiment. Stimuli were measured by photovoltaic stimulus isolation units (Grass PSIU6). The resulting voltage deflections were measured differentially by a pair of extracellular glass microelectrodes (filled with low [Cl⁻] ACSF) positioned between the two stimulation electrodes. All four electrodes were placed in a straight line in stratum (st.) radiatum; distance of the two stimulation electrodes was at ~1 mm, and that of the recording electrodes was at ~500 μm (Fig. 6A). The amplitudes of the voltage deflections were determined at the steady-state level, and changes in electrical tissue resistance were normalized to the prehypoxic tissue resistance of the respective slice in normal solution. Because the electrode distances varied somewhat in the different slices, only these relative changes are reported.

**Recording of the intrinsic optical signal**

Light reflectance or transmittance was recorded in st. radiatum of the CA1 region using a 12-bit charge-coupled device (CCD) camera (Princeton Instruments, PentaMAX System). Slices were illuminated by white light at angles of 20° relative to the line of vision of the microscope objective (reflectance), or 180° (transmittance). Size of the digitized pictures was 248 × 190 pixel; their optical resolution was 45 μm/pixel. Images were recorded at 3-s intervals and 0.2-s exposure time and transferred to an IBM-compatible computer. Hypoxia-induced changes were visualized by digital image subtraction and were referred to the image taken before SD onset. The optical changes were displayed in a 256 grayscale-scale mode covering a full-scale range of ±15% brightness changes and quantified for a representative rectangular region of interest close to the microelectrode recording DC potential and/or TMA⁺ signal (Fig. 2B). The initial time course of the IOS was found to be linear until the maximum brightness was approached (1st 30–40 s). We therefore fitted the relative changes during the first 30 s (1st 10 images) after onset of the reflectance increase by linear regression and used the resulting slope as a measure for the invasion speed of the CA1 region. Photoshop 4.0 software (Adobe) was used for graphic processing.

**Statistics**

The data were obtained from 98 rats. Because most experiments lasted between 4 and 8 h, usually only one slice could be used from each brain. Numerical values are represented as means ± standard deviation. Significance was tested using a two-tailed, unpaired Student’s t-test, and a significance level of 5%. In the diagrams, significant changes are marked by asterisks (*P < 0.05; **P < 0.01). Statistical calculations and linear regressions were done with the Excel 7.0 or QuattroPro 3.0 software.

**RESULTS**

**Electrical signs of SD in different regions of hippocampal slices**

The hippocampal CA1 region is highly vulnerable to oxygen withdrawal and has a low threshold for SD initiation. Simultaneous recording of DC potential changes during severe hypoxia from different hippocampal areas showed that hypoxic SD can occur in all hippocampal regions, although it occasionally spared CA3 (Fig. 1). It was always most prominent in CA1 and the adjacent subiculum; a spread from CA1/CA2 into CA3, or vice versa, was never observed. Mean amplitudes for the rapid negative DC potential deflection (ΔVₜ) were as follows: CA1, −14.5 ± 7.3 mV; CA2/3, −6.3 ± 5.4 mV; CA3, −6.5 ± 7.5 mV; CA4, −6.9 ± 6.1 mV; dentate gyrus, −7.8 ± 6.0 mV; subiculum, −15.3 ± 7.9 mV; (mean ± standard deviation; each n = 19).

**Optical changes**

Mapping the hypoxia-induced increase in light reflectance to the various slice regions of a different set of 37 control slices showed that severe hypoxia induced optical changes in the CA1 region of every slice investigated. In 17 of 37 slices, the IOS spread from the CA1 area into the subiculum, but only in one slice did it enter the dentate region. Optical changes in CA2 occurred in 11 of the investigated slices, but a further spread from CA1 into CA3 could never be observed (see also Aitken et al. 1998, 1999; Müller and Somjen 1998c; Obeidat and Andrew 1998). In some slices, however, an IOS appeared in CA3 independently from that seen in CA1. Because the electrical and optical signs of hypoxic SD usually occurred first and were most intense in CA1 region, in many of the trials we limited the optically recorded field to the CA1 region to improve spatial resolution.

In the CA1 region of interfaced rat hippocampal tissue slices, severe hypoxia induced biphasic changes in light reflectance at the slice surface. In all slices, hypoxic SD was preceded by a slowly progressing reflectance decrease, which continued until SD onset. This slow decrease was replaced by a sudden, large increase in light reflectance, which coincided exactly with the hypoxic ΔVₜ onset beginning 1.5–3 min after oxygen withdrawal (Fig. 2A) (see also Aitken et al. 1998; Müller...
Recovery of the optical changes was much slower within 45 s. Under control conditions the reflectance increase in st. radiatum averaged (Aitken et al. 1998; Müller and Somjen 1998c). The reflectance increase started in a small region. It was most pronounced in st. radiatum, followed by st. oriens, whereas st. pyramidale was mostly spared (Fig. 2B) (Aitken et al. 1998; Müller and Somjen 1998c; Snow et al. 1983). Because Cl⁻ fluxes via either Cl⁻ channels or secondary active Cl⁻ transport systems play a key role in volume regulation and cell swelling (for review see Kaplan et al. 1996; Strange 1993), we investigated whether the established Cl⁻ transport inhibitors furosemide, DIDS, and DNDS as well as Cl⁻ substitution by methylsulfate modulate the intrinsic optical signals associated with hypoxic SD.

The inhibitors and the Cl⁻ substitute were applied for 45 min before hypoxic SD was induced. In well-oxygenated slices their administration hardly affected the light reflectance baseline. Although in untreated control slices the tissue reflectance decreased during 45 min by 0.64 ± 1.01% (n = 8), Cl⁻ transport inhibition caused the following reflectance changes: furosemide, −0.28 ± 1.55% (n = 7); DIDS, 1.74 ± 1.3% (n = 6); DNDS, 0.32 ± 1.49% (n = 8); Cl⁻ substitution by methylsulfate, 0.19 ± 2.02% (n = 8).

During SD and hypoxic SD the extracellular Cl⁻ concentration drops by at least 60 mM (Nicholson 1984; Nicholson and Kraig 1981), due to Cl⁻ influx into neurons and glial cells (Jiang et al. 1992). Accordingly, we observed distinctive changes in both electrical and optical signs of hypoxic SD, when hypoxia was induced in the presence of the Cl⁻ transport inhibitors or the Cl⁻ substitute. We have previously found that furosemide and DIDS depress the amplitude of the hypoxic ΔVₒ by 19 and 33%, respectively (Müller and Somjen 1998a,b). Similarly to its effect on ΔVₒ, furosemide also depressed the hypoxia-induced intrinsic optical signal. The intensity of the reflectance increase was reduced by 28.6 ± 14.0%, and it evolved 34.3 ± 14.1% slower (n = 8, Fig. 3B). DIDS and DNDS (0.1 mM) did not affect hypoxia-induced intrinsic optical signals (n = 7 and n = 5, respectively).

The most remarkable effect occurred when 98% of extracellular Cl⁻ was replaced by the membrane-impermeable meth-
FIG. 2. Onsets of extracellular DC potential shift and reflectance increase coincide during hypoxic spreading depression (SD). A: simultaneous recordings of extracellular DC potential ($\Delta V_o$) and light intensity reflected from the slice surface. Note simultaneous start but different time courses of the 2 traces. Arrows below the reflectance plot indicate the times at which the images of B were recorded. The site of the microelectrode in st. radiatum and the "region of interest" in which reflected light intensity was measured are shown in the 1st image of B. B: subtraction images showing hypoxia-induced changes in light reflectance. Reflectance increases 1st at the CA1/subiculum border then spreading toward the CA1/CA2 border. Numbers above the images indicate time since oxygen withdrawal. Note that the optical changes are most pronounced in st. orients (so) and st. radiatum (sr), but mostly spare st. pyramidale. The gray-scale represents brightness changes as % of baseline intensity. The image area is approximately $1.8 \times 1.3$ mm.

FIG. 3. Optical signal intensity changes during repeated hypoxia in normal solution and during furosemide administration and Cl$^-$ substitution. A: hypoxia induced at 45-min intervals under control conditions; plotted are the average light reflectance changes of 8 slices, normalized to the maximal reflectance increase (1.0) during the 1st (control) SD. Zero ordinate represents light intensity at the onset of SD, and the abscissa shows time in minutes with 0 being the onset of SD-related $\Delta V_o$. Note the initial reflectance decrease before SD onset. B: furosemide (2 mM) decreased and slowed the optical changes. Wash out (45 min) failed to reverse these effects ($n = 8$). C: replacing extracellular Cl$^-$ by methylsulfate reversibly suppressed the SD associated reflectance increase, which was replaced by a decrease of reflectance ($n = 6$). D: similar to C but showing light transmittance. The SD-associated decrease in light transmittance was also reversibly suppressed by Cl$^-$ withdrawal and was replaced by increased light transmittance during SD ($n = 5$).
In methylsulfate-substituted solution the amplitude of the hypoxic \( \Delta V_o \) decreased by only 32.8 \( \pm \) 11.2\% \((n = 11)\), but the SD-associated reflectance increase was completely suppressed. Instead, hypoxic SD was now paralleled by a smaller but still substantial light reflectance decrease, which was 76.3 \( \pm \) 17.5\% less intense than the reflectance changes seen under control conditions \((n = 6;\) Figs. 3C and 4). Correspondingly, the decrease in light transmittance during SD was also reversed in methylsulfate-based low Cl\(^-\) solutions, and it was replaced by brightening of the slices in transmitted light during SD, being 76.2 \( \pm \) 55.1\% less intense than the previously recorded transmittance decrease in ACSF \((n = 5,\) Fig. 3D). Both the \( \Delta V_o \) depression and the inversion of the optical signs of SD by Cl\(^-\) withdrawal were completely reversible; 90 min after readdition of Cl\(^-\) hypoxic SD increased light reflectance by 126.5 \( \pm \) 29.8\% of control \((n = 6)\), and light transmittance decreased by 263.1 \( \pm \) 126.6\% of control \((n = 5;\) Figs. 3, C and D, 4). Substitution of Cl\(^-\) by gluconate also consistently and reversibly abolished the reflectance increase during SD and replaced it by a reflectance decrease \((n = 5)\).

**TMA\(^+\) space changes compared with the optical signals in normal solution and after Cl\(^-\) withdrawal**

Because the Cl\(^-\) dependence of the optical changes suggested that cell swelling could be responsible for the generation of the IOS, we simultaneously recorded interstitial TMA\(^+\) concentration \([\text{TMA}^+]_o\), \( \Delta V_o \), and light reflectance. Because a constant extracellular Cl\(^-\) concentration was an absolute requirement (especially in low Cl\(^-\) solutions), we were not able to apply TMA\(^+\) by either pressure of iontophoretic ejection (Dietzel et al. 1980; Nicholson 1992; Nicholson and Phillips 1981) and were methodologically restricted to the...
analysis of TMA<sup>+</sup> background changes. These measurements were first performed in normal [Cl<sup>-</sup>]<sub>o</sub> solution and then repeated on the same slice 45 min after substitution of extracellular Cl<sup>-</sup> by methylsulfate, and again 45 min after restoring [Cl<sup>-</sup>]<sub>o</sub>. As shown in Fig. 2B, the reflectance changes were analyzed in a region close to the recording electrode.

During the initial phase of hypoxia, before onset of the SD-like ΔV<sub>e</sub>, a slow negative DC potential shift, a slight decrease in interstitial volume (−9.5 ± 3.6%), and a decrease in light reflectance (decreased scattering) occurred simultaneously. In normal [Cl<sup>-</sup>]<sub>o</sub>, hypoxic SD began 1.4 ± 0.4 min after oxygen withdrawal. It was indicated by the sudden ΔV<sub>e</sub> of −18.2 ± 5.3 mV, and it coincided with a 63% [TMA<sup>+</sup>]<sub>o</sub> increase (corresponding to a reduction of interstitial space by 37.2 ± 9.5%) and an 9.8 ± 4.1% (n = 13) increase in light reflectance. Both the optical changes and the cell swelling evolved more slowly than the ΔV<sub>e</sub>. Although the ΔV<sub>e</sub> reached its peak within 2.8 ± 3.3 s, cell swelling and reflectance increase reached their maximum intensity 35.4 ± 22.2 s and 49 ± 17 s (n = 13) after SD onset. Measured at the half-amplitude level, optical changes and [TMA<sup>+</sup>]<sub>o</sub> increase lasted 107 ± 44 s and 81.8 ± 26.8 s, respectively, compared with 33.7 ± 10.9 s (n = 13) for the ΔV<sub>e</sub>. During the posthypoxic recovery phase, [TMA<sup>+</sup>]<sub>o</sub> usually undershot the baseline by 13.7 ± 6.9% (Fig. 5A), which probably results from posthypoxic cell shrinkage. It could also indicate loss of TMA<sup>+</sup> from tissue spaces during the preceding period of [TMA<sup>+</sup>]<sub>o</sub> elevation, by diffusion of TMA<sup>+</sup> into unaffected tissue areas and the bath.

To estimate exchange between tissue and bath, two types of
experiments were conducted. In one series, hypoxia was maintained for 15 min after SD onset. During such prolonged hypoxia, \([\text{TMA}^+]_o\) gradually declined by 38.0 \(\pm\) 11.5% \((n = 5)\). Assuming that the swollen state of cells was maintained until reoxygenation was started, this slow decline represents loss of \(\text{TMA}^+\) from the tissue to the bath. As soon as oxygen was readmitted, the decline of \([\text{TMA}^+]_o\) toward baseline was markedly accelerated. In a second set of trials, bath \([\text{TMA}^+]\) was raised and then lowered while tissue \([\text{TMA}^+]\) was recorded in the presence of normal oxygen. TMA equilibration in well-oxygenated slices was faster than in hypoxic slices. The slower diffusion in the hypoxic tissue is explained by the restricted diffusion path due to cell swelling. In slices previously incubated for 45 min in a bath containing 3 mM \(\text{TMA}^+\), the half-decay time for reequilibration in 1.5 mM \(\text{TMA}^+\) medium averaged 204 \(\pm\) 40.3 s \((n = 5)\). By contrast, topical application of 3 mM \(\text{TMA}^+\) ACSF resulted in shorter half-decay times \((99.2 \pm 52.4, n = 6)\), indicating, as expected, that equilibration with the bath is much slower when \([\text{TMA}^+]_o\) is raised diffusely in wide areas or the entire slice, as was the case in the trials involving hypoxic SD.

Because withdrawal of extracellular \(\text{Cl}^-\) caused the reversal of the optical signs of SD (Figs. 3 and 4), we now asked whether it also prevented cell swelling. In the presence of normal oxygen, substitution of extracellular \(\text{Cl}^-\) by methylsulfate transiently widened \(\text{TMA}^+\) space by 9.0 \(\pm\) 5.0% \((n = 8)\), following which \([\text{TMA}^+]_o\) returned to its baseline. This slow apparent recovery was probably in large part due to diffusion of \(\text{TMA}^+\) from the bath into the tissue, with a possible contribution of volume regulation especially in glial cells. On restoration of \([\text{Cl}^-]_o\), the calculated extracellular space decreased from its perhaps still widened volume by 19.1 \(\pm\) 18.5% \((n = 6)\).

Contrary to expectation, when hypoxic SD was induced 45 min after \(\text{Cl}^-\) had been replaced by methylsulfate, the hypoxia-induced decrease in calculated \(\text{TMA}^+\) space was not significantly affected, averaging 101.9 \(\pm\) 26.6% of control \((n = 8;\) Figs. 5B and 6C) However, an undershot of the \([\text{TMA}^+]_o\) baseline during the recovery phase did not occur. In these slices, as before, the SD-related reflectance increase was suppressed and replaced by reflectance decrease, whereas the \(\Delta V_o\) amplitude decreased by 31.2 \(\pm\) 9.5%. When hypoxic SD was induced 45 min after restoring \([\text{Cl}^-]_o\), the \(\Delta V_o\) amplitude averaged −12.7 \(\pm\) 4.3 mV, a reflectance increase of 8.7 \(\pm\) 3.5% was again observed and extracellular space decreased by 88.8 \(\pm\) 24.4% of control \((n = 8)\).

The unchanged amount of cell swelling in low \(\text{Cl}^-\) solutions might indicate that the cell membranes became permeable for methylsulfate during hypoxia. We therefore repeated these experiments using the even less permeable \(\text{Cl}^-\) substitute, gluconate. Administration of gluconate-based low \(\text{Cl}^-\) solution reversibly widened \(\text{TMA}^+\) space by 11.0 \(\pm\) 1.8% \((n = 5)\). In the presence of gluconate, the hypoxic \(\Delta V_o\) amplitude decreased by 33.3 \(\pm\) 18.4%, and SD occurred 33.5 \(\pm\) 24.7% \((n = 5)\) earlier than under control conditions. In the same five slices the SD-associated reflectance increase inverted into a 79.2 \(\pm\) 13.8% less intense reflectance decrease, similar to the effect of methylsulfate. Again, the increase in \([\text{TMA}^+]_o\) was not suppressed, averaging 103.0 \(\pm\) 5.6% of control and indicating unchanged cell swelling (Fig. 6C). An undershot of the

FIG. 6. Changes in electrical tissue resistance during hypoxic SD. A: diagram of the electrode array. B: sample recording of extracellular potential \((V_o)\) and normalized resistance changes \((R_o)\). As the voltage was measured between 2 glass microelectrodes in the tissue, the SD-related DC deflection is biphasic. The vertical voltage deflections were generated by the applied constant current pulses and were used to derive the \(R_o\) values. C: comparison of \(R_o\) increase and \(\text{TMA}^+\) space decrease during hypoxic SD in control solutions and following \(\text{Cl}^-\) substitution by methylsulfate and gluconate, normalized to the change in control solution. Although the \(\text{TMA}^+\) signal was not reduced by either of the \(\text{Cl}^-\) substitutes \((n = 8\) for methylsulfate, \(n = 5\) for gluconate), the increase in tissue resistance was dampened to 57.7% of control in methylsulfate solutions \((n = 6)\) and was almost suppressed in gluconate solutions \((n = 5)\). TMA\(^+\) signal and tissue resistance in the presence of gluconate were simultaneously recorded in the same slices. Error bars represent standard deviations \((^{*}P < 0.05; ^{**}P < 0.01)\).
[TMA\(^+\)]\(_o\) baseline during the recovery phase was not observed.

**Changes in electrical tissue resistance during hypoxic SD**

To confirm the unexpected observation that cell swelling still occurs following nearly complete Cl\(^-\) substitution, we measured the electrical tissue resistance during hypoxic SD in normal and low Cl\(^-\) solutions (Fig. 6). Restriction of extracellular space during SD is well known to increase the tissue resistance (Hoffman et al. 1973; Marshall 1959; Van Harreveld and Ochs 1957), even though the input resistance of neurons, and to a lesser degree that of glial cells, decreases. In our experiments, hypoxic SD in control solution was associated with an increase in tissue resistance by 35.5 ± 10.1% (n = 11). The time course of the resistance changes resembled the time course of the [TMA\(^+\)]\(_o\) changes, and following reoxygenation the tissue resistance also undershot the prehypoxic baseline level (Fig. 6B). In normal oxygen, Cl\(^-\) substitution by methylsulfate and restoration of normal [Cl\(^-\)]\(_o\) did not significantly affect the electrical tissue resistance, which averaged 108.2 ± 17.6% and 93.9 ± 24.3% of control (n = 6), respectively. When hypoxic SD was induced in methylsulfate-containing solution, the tissue resistance still clearly increased, the increase averaging 57.7 ± 11.0% of control (n = 6), which confirms that Cl\(^-\) substitution does not abolish cell swelling during severe hypoxia. After restoration of [Cl\(^-\)]\(_o\), the increase in tissue resistance during SD averaged 113.0 ± 34.7% of control (n = 6).

Cl\(^-\) substitution by gluconate had, however, a different effect on tissue resistance. On administration of gluconate, the tissue resistance at first became almost double and then decreased again somewhat, averaging after 45-min treatment 126.5 ± 5.4% of control. During hypoxic SD the tissue resistance increase was abolished, averaging only 2.4 ± 1.6% of control (n = 5). Yet the increase in [TMA\(^+\)]\(_o\), measured in the same slices with the TMA\(^+\)-sensitive electrode positioned in between the two extracellular recording electrodes, was not diminished (108.4 ± 5.8% of control; n = 5). Restoring normal [Cl\(^-\)]\(_o\), decreased the tissue resistance to 99.2 ± 7.0% of its control level and during hypoxia tissue resistance increased again, by 93.0 ± 28.5% (n = 5) of control, whereas [TMA\(^+\)] increased by 84.4 ± 11.4% of control.

**Contribution of glial cells**

Severe hypoxia caused the most prominent scattering changes in st. oriens and st. radiatum, i.e., in layers rich in glial processes. To decide whether glial cells contribute to the generation of the optical signals, we impaired glial function by metabolic poisoning with fluorooacetate. Fluorooacetate is selectively taken up by glial cells and metabolized into fluorocitrate, which arrests the glycoltricarboxylic acid cycle (citric acid cycle) and thus blocks glial metabolism (Clarke and Nicklas 1970; Hassel et al. 1992). Over the course of 3–4 h, hippocampal glial cells gradually depolarize, [K\(^+\)]\(_o\) slightly increases, and pH\(_o\) decreases. Neurons are, however, at first almost completely unaffected and severe neuronal damage expressed as loss of electrogenic activity does not occur before 8 h of fluorooacetate treatment (Largo et al. 1996, 1997a). In our experiments application of 5 mM fluorooacetate for up to 6 h shortened the time to onset of hypoxic ΔV\(_o\). The reduction in time to SD onset averaged 20.6 ± 8.3% after 1.5 h and 28.9 ± 21.6% after 6 h fluorooacetate treatment, whereas ΔV\(_o\) amplitude and duration were not significantly affected (n = 9; Figs. 7 and 8A). The intensity of the SD-associated scattering increase was greatly enhanced, reaching after 6 h fluorooacetate treatment 296.6 ± 197.1% of its control amplitude (n = 6; Figs. 7 and 8B).

**Failure to demonstrate a role of mitochondrial swelling in IOS generation**

Hypoxia is known to induce the mitochondrial permeability transition and result in mitochondrial swelling (Lemasters et al. 1997). The trigger event of mitochondrial swelling appears to be the activation of the so-called “megachannel” in the outer mitochondrial membrane, which enables anion and cation fluxes into the mitochondrion (Colombini 1994; Lemasters et al. 1997). Interestingly, mitochondrial swelling is highly Cl\(^-\) dependent (Azzzone et al. 1976a,b), and it has been reported to cause scattering changes in hippocampal slices (Johnson et al. 1998). We therefore attempted to test whether blockers of the mitochondrial permeability transition can prevent the hypoxia-induced scattering increase. Application of 5 μM cyclosporin A (60 min) an inhibitor of the mitochondrial “megachannel” (Colombini 1994; Lemasters et al. 1997; Qian et al. 1997) did not significantly affect the hypoxic ΔV\(_o\) amplitude by 25.0 ± 20.8% and shortened its duration by 21.2 ± 16.5%. It failed, however, to depress the SD associated reflectance increase (n = 5). Raising extracellular Mg\(^2+\) to 5 mM (Kristal and Dubinsky 1997) reversibly delayed the onset of the hypoxic ΔV\(_o\) by 46.0 ± 33.3% and decreased focal excitatory postsynaptic potential (fEPSP) amplitudes by 58.5 ± 26.5%, but again the SD associated scattering increase was not reduced (n = 7).

**No evidence for the involvement of ATP-sensitive K\(^+\) channels in SD or its IOS**

The involvement of ATP-sensitive K\(^+\) channels in the hypoxic hyperpolarization of CA1 pyramidal neurons preceding hypoxic SD is controversial (Erdemli et al. 1998; Fujimura et al. 1997). Modulation of these channels by 45-min applications of the specific inhibitor glibenclamide (0.1 mM; n = 7) or the activator diazoxide (0.1 mM; n = 6) did not affect the hypoxic ΔV\(_o\) or the associated optical signals. Therefore these channels are unlikely to play a crucial role in the triggering of hypoxic SD or the generation of the IOS.

**Discussion**

**Dual nature of hypoxia-induced IOSs**

Intrinsic optical signals associated with severe hypoxia are characterized by biphasic optical changes. An initial decrease in reflected light changes at the onset of the SD-like event to a much more pronounced reflectance increase. Accordingly, when transmitted light was recorded, an initial increase in transmittance was followed by a transmittance decrease (Fig. 3). Because, during both phases, reflectance and transmittance were consistently influenced in opposite sense, these optical signals may be attributed to changes in light scattering within the tissue.
Kreisman et al. 1995 described an inversion of the sign of transmitted light change that depended on the bath level, the signal changing as the slice was either submerged in solution or exposed at the upper surface to the gas phase. In our experiments the level of the bath was not changed, and the slices were constantly located at the liquid/gas interface; therefore these changes in experimental conditions cannot explain the reversal of the optical changes that coincides with SD onset. It could be argued, however, that during the first few minutes of hypoxia cell swelling was mild and the dominant IOS was reduced scattering within the tissue, whereas during SD the severe swelling caused the surface of the slice to bulge, and the “lensing” effect described by Kreisman et al. (1995) became dominant. This is also unlikely, because a reduced radius of curvature would have diminished the intensity of both, reflected and transmitted light (see INTRODUCTION) but, in fact, reflected light consistently increased during SD in normal solution, in agreement with earlier reports (Aitken et al. 1998; Martins-Ferreira and Oliveira Castro 1966; Müller and Somjen 1998c; Snow et al. 1983). We therefore conclude that during the initial, pre-SD phase of hypoxia light scattering decreases, whereas SD itself coincides with a marked increase in scattering within the tissue.

Dissociation of electrical signals, IOS and cell swelling following Cl− withdrawal

Although the sudden hypoxic ΔV_o, the scattering increase, and the restriction of extracellular space coincided in control solutions, these measures were differently affected when hypoxia was induced following Cl− substitution. The extracellular potential shift was partially depressed, and the scattering increase was completely suppressed, but cell swelling was unaffected.

The persistence of the hypoxic ΔV_o in low Cl− solutions and the fact that SD-related depolarization of pyramidal neurons is not reduced by Cl− withdrawal (Müller and Somjen 1998b) indicate that Cl− fluxes are not responsible for the massive potential shifts or their regenerative nature. The observed 31% decrease in ΔV_o amplitude (Figs. 4 and
5) is similar to that reported by Do Carmo and Martins-Ferreira (1984) for normoxic SD in isolated retina. It could be due to the moderate widening of extracellular space following Cl\(^-\) withdrawal (as indicated by the decrease in \([\text{TMA}^+]\)\(_o\)), which may dampen extracellular potential shifts, or desynchronized neuronal depolarization as was reported for epileptiform discharges in the presence of furosemide (Hochman et al. 1995).

The most significant observation was, however, the separation of SD-related scattering increase and cell swelling. Contrary to expectation, after Cl\(^-\) withdrawal, SD-related cell swelling, measured as the \([\text{TMA}^+]\)\(_o\) increase, was unchanged. Even though it is an indirect measure, the indicator dilution method is a well-established technique (Dietzel et al. 1980; Hansen and Olsen 1980; Nicholson 1992; Nicholson and Phillips 1981; Phillips and Nicholson 1979). Of the different possible marker ions (TEA, TMA, \(\alpha\)-naphtalenesulfonate, choline), TMA\(^+\) has been deemed to be the best (Nicholson 1992), even though it was occasionally reported that TMA\(^+\) may sometimes enter neurons and glial cells (Ballanyi et al. 1990; Jing et al. 1994; Nicholson 1992), thereby causing an underestimation of volume changes.

Unlike the TMA\(^+\) signal, the well-known SD-related increase in tissue resistance (Marshall 1959; Van Harreveld and Ochs 1957) was reduced when methylsulfate replaced Cl\(^-\), and even more when the substitute was gluconate. The question is, which measurement, TMA\(^+\) or resistance, is the more reliable. For the following reasons we believe that TMA\(^+\) space gives a better indication. Tissue resistance can be an accurate indicator of cell volume only as long as the applied current flows in interstitial space and not through cells. Okada et al. (1994) have shown that, in the turtle cerebellum, a considerable fraction of the test current in fact does flow through the transcellular path. Although the proportion undoubtedly varies among tissues, this source of error cannot be neglected. The error becomes worse, if the interstitial resistance increases while transcellular resistance does not. We found that the resistivity of methylsulfate-based ACSF is \(~30\%\) higher than that of normal solution, and for gluconate-based ACSF the resistivity increase was 73\%. Moreover, with normal oxygen supplied, both methylsulfate and gluconate substitution of Cl\(^-\) caused the prehypoxic interstitial volume to increase by 9–10\%. This shifts the resistance ratios in the “voltage divider” and as a result an equal (absolute) increase of cell volume would restrict the fractional interstitial volume to a (relatively) lesser degree. It should be noted that exposure to hyperosmotic solutions also results in a much greater increase in TMA\(^+\) space than decrease of tissue resistance, and it is the TMA\(^+\) signal that correlates with the depression of synaptic transmission (Huang and Somjen 1995).

The degree of cell swelling during hypoxic SD measured here (37\% ISV restriction in st. radiatum) was less pronounced than that reported by Jing and coworkers (1994; their value: 66\% ISV restriction in st. pyramidalis). This discrepancy may be due to the different cytoarchitectonic layers investigated or technical differences. Because we based estimates on baseline \([\text{TMA}^+]\)\(_o\) instead of repeated iontophoretic ejection of the indicator, diffusion of TMA\(^+\) during its elevated level into tissue areas not affected by SD and into the bath might have resulted in an underestimation of cell swelling.
Possible mechanisms of cell swelling in low Cl\(^{-}\) solution

If we accept that the TMA\(^{+}\) signal accurately indicates the fractional interstitial volume, it appears that the prominent cell swelling during hypoxic SD was not prevented by Cl\(^{-}\) withdrawal. However, the absence of an undershoot of the [TMA\(^{+}\)]\(_{0}\) baseline following SD recovery in low Cl\(^{-}\) solutions might indicate impairment of regulatory volume decrease in the presence of methylsulfate and gluconate.

The unchanged amount of cell swelling is surprising because there can be little doubt that, in the presence of normal [Cl\(^{-}\)]\(_{0}\), hypoxic cell swelling depends on Cl\(^{-}\) uptake into cells (Strange 1993; Van Harreveld 1966). We must ask what drives the influx of water in the absence of chloride. It appears unlikely that the remaining [Cl\(^{-}\)]\(_{0}\) (3.9 mM) is sufficient to account for the unchanged amount of cell swelling. Because most Cl\(^{-}\) channels are also permeable to bicarbonate, the observed cell swelling in low Cl\(^{-}\) solutions could be due to bicarbonate influx into neurons and glial cells, which of course would disturb intracellular pH. Additional but less likely factors may be enhanced membrane permeability to larger anions during SD, permitting entry of methylsulfate and gluconate. Phillips and Nicholson (1979) determined the penetration of several anions during normoxic SD in cerebellum, but methylsulfate and gluconate were not among those tested. Products of anaerobic metabolism could raise the osmolarity of the cytosol, but this effect is probably too slow to explain the explosive SD-like swelling. Even less probably, interstitial space could have shrunk without cell swelling, in the manner of a squeezed sponge, but no force is known that would be able to perform the squeezing.

Unlike SD-related cell swelling, stimulation-induced volume changes in neocortical slices are abolished when Cl\(^{-}\) is substituted by gluconate (Holthoff and Witte 1996). Holthoff and Witte (1996) attributed the swelling mainly to uptake of KCl, probably by glial cells. Because fluoroacetate and fluoroacetate do not diminish electrophysiological signs of normoxic or hypoxic SD (Largo et al. 1997a,b) (and see results in this paper), it seems likely that SD-related swelling resides mainly in the dendritic arbor of neurons. It seems therefore that high K\(^{+}\)-induced glial swelling is Cl\(^{-}\)-dependent, whereas SD-related neuronal swelling is not. Apparently at variance with our observations, Lipton (1973) reported that hypoxia-induced reflectance changes were abolished when Cl\(^{-}\) was substituted by gluconurate. In the absence of electrophysiological recording it is, however, doubtful that his McIlwain-style submerged slices underwent SD-like depolarization. Gradual cell swelling and depolarization can occur without the SD-like event, and the slow swelling observed by Lipton (1973) may be Cl\(^{-}\)-dependent, whereas the abrupt SD-related swelling is not.

At least two mechanisms induce light-scattering changes

Intrinsic optical signals caused by neuronal excitation are about an order of magnitude less intense than the optical changes associated with SD. The most remarkable difference is, however, that they show opposite direction. Although neuronal excitation decreases light scattering, SD is paralleled by a scattering increase. The Cl\(^{-}\)-dependence of the decrease of light scattering and its sensitivity to furosemide have already been reported (Hochman et al. 1995; Holthoff and Witte 1996; Lipton 1973; MacVicar and Hochman 1991) and were taken to support the association of these signals with cell swelling. The results reported here show that this is not the case for the scattering increase associated with hypoxic SD. Cl\(^{-}\)-withdrawal caused the separation of the electrical and optical signs of SD. The reflectance increase was completely suppressed, whereas cell swelling was not affected (Fig. 5).

Furthermore, st. radiatum and st. pyramidale show a comparable amount of cell swelling during SD (Jing et al. 1994; Pérez-Pinzón et al. 1995), but increased scattering occurs only in the dendritic layers. We therefore conclude that cell swelling and increased light scattering are not correlated.

The evidence is compelling, however, that the reduced scattering seen in mild to moderate hypotonia and in response to electrical stimulation of the tissue is indeed related to increased cell volume (Aitken et al. 1999; Andrew and MacVicar 1994; Holthoff and Witte 1996; Lipton 1973; MacVicar and Hochman 1991). Kreisman et al. (1995) reported, however, that, unlike in submerged slices, in slices at a liquid-gas interface light transmittance decreased with reduced osmolarity. This was not the case in our interface chamber where withdrawal of 40 mM NaCl decreased light reflectance by 2.9 ± 2.1% (n = 10, data not shown) indicating decreased scattering. In a different study hypotonic exposure increased transmittance and decreased reflectance of hippocampal slices in two interface chambers with differing optical systems, as long as osmolality was reduced by not more than 75 mosm/kg (Aitken et al. 1999; D. Fayuk, unpublished observations). It therefore seems very likely that the mild decrease of light scattering seen during hypoxia before SD, as well as the much more marked scattering decrease observed during SD in low Cl\(^{-}\) solutions (Figs. 3 and 4), are caused by cell swelling. Surprisingly, we also found (Aitken et al. 1999) that the decreased light scattering typical of mild to moderate hypotonia inverted into an increase when bath osmolarity was reduced to almost half normal (~14 mosm/kg). Recurrent waves of SD induced by the very low osmolarity were accompanied by waves of scattering increase on an already elevated baseline.

We conclude that there are at least two processes that influence light scattering in opposite sense. The dilution of macromolecules in the cytosol during cell swelling is probably responsible for the reduction of light scattering consistently observed in cell suspensions and tissue slices exposed to mild to moderate hypotonicity, as well as in neural tissue stimulated electrically. A second process of unknown origin causes an increase in light scattering, and it appears to dominate when hippocampal slices are exposed to severe hypotonicity or undergo SD-like depolarization. Removal of Cl\(^{-}\) abolishes the second process and unmasks the first, revealing that the swelling-induced scattering decrease is latently present even during hypoxic SD.

Possible generators of the light-scattering increase

Glia poisoning by fluoroacetate (Clarke and Nicklas 1970; Hassel et al. 1992) did not prevent the initiation and the spread of hypoxic SD but rather enhanced the tissue’s susceptibility to SD, as has already been reported for normoxic SD (Largo et al. 1997a,b). The progressive impairment of glial function gradually increased the intensity and extension of the light-scattering increase during SD, until it reached threefold intensity after 6 h.
(Figs. 7 and 8). We therefore conclude that generation of the SD-associated scattering increase does not require intact glial cells or a functioning glial “syncytium.” The huge increase of the SD-related optical signal after glial poisoning suggests that intact functioning glia dampens the process that causes the scattering increase. In any event, neurons and not glial cells appear to be mostly responsible for the scattering increase.

The predominance of SD-related optical changes in st. radiatum and st. oriens, as well as their persistence during glial poisoning do suggest that the underlying mechanisms occur mainly in the dendritic neuronal processes, or the interstitial space in between. Even though the refraction by cell membranes embedded between cytosol and interstitium may be minor, when many fine processes are closely packed, their scattering effect may be multiplied. A measure of geometric complexity of interstitial space is the tortuosity (Nicholson and Phillips 1981). Pérez-Pinzón et al. (1995) have determined tortuosity as well as interstitial volume fraction during oxygen and glucose withdrawal in hippocampal slices. Tortuosity in the pyramidal layer of CA1 region did not significantly change during SD-like depolarization, and this suggests that it did not contribute to the optical signal. Because, however, st. radiatum was not explored by Pérez-Pinzón et al. (1995), an effect by tortuosity cannot be ruled out. In fact increased tortuosity may even be suggested by the hindered TMA+ diffusion observed during maintained hypoxia.

The scattering increase is not due to the bulging or “lensing” of the swollen slice surface reported by Kreisman and coworkers (1995), first, because cell swelling was unaltered after Cl− withdrawal while the scattering increase was reversibly abolished and, second, because bulging of the surface should alter reflectance and transmittance in the same sense (see introduction). Besides, the strong increase of reflectance during SD in submerged retinae (Martins-Ferreira and Oliveira Castro 1966) confirms that scattering can increase in neural tissue independently from surface optics. In the submerged slices maintained at 33–34°C by Basarsky et al. (1998), the scattering increase was, apparently, not activated during SD. Polischuk and colleagues (1998) and Obiedat and Andrew (1998) report that light transmittance decreases in such slices when oxygen and glucose are withdrawn at 37.5°C, and they relate this change from increased to decreased transmittance (i.e., from decreased to increased scattering) to irreversible damage, expressed as beading of neuronal dendrites. Because in our experiments the SD-related scattering increase evolved and reversed rapidly without permanently impairing synaptic or other neuronal function, dendritic injury cannot be its cause.

The most obvious two sources of light scattering in live tissue are macromolecules in cytosol, and cell organelles. Influx of water into cells dilutes macromolecules and is the likely reason for the decreased scattering during swelling. Swelling of organelles, on the other hand, would increase the surfaces of scattering particles, and it could be the mechanism of Cl−-dependent scattering increase. As a possibility we investigated the involvement of mitochondrial swelling, which is triggered by hypoxia (Lemasters et al. 1997) and may induce scattering changes in hippocampal slices (Johnson et al. 1998). Application of cyclosporin A and elevated [Mg2+]o, effectively reduce or even prevent mitochondrial swelling in isolated mitochondria or permeabilized cells (Kristal and Dubinsky 1997; Qian et al. 1997). In our experiments, however, these agents failed to reduce the SD-associated optical signals. Even though the shortened SD duration (−21.2 ± 16.5%) may suggest a protective effect of cyclosporin A, its penetration into the tissue slice and uptake into cells are, however, questionable and leave the outcome inconclusive. Besides, it is not clear whether mitochondrial swelling is already triggered immediately after SD onset or whether it is the consequence of continued hypoxia only.

Besides mitochondrial swelling and beading of dendrites, hypoxia and ischemia are known to induce a variety of ultrastructural changes, such as chromatin clumping and nucleolar condensation, cytoskeletal and microtubule breakdown resulting in cell shape changes, membrane blebbing and increased membrane fluidity as well as disruption of endoplasmic reticulum and Golgi apparatus (Allen et al. 1989; Kwei et al. 1993; Tanaka et al. 1999). Each of these changes could modify light scattering, but it should be considered that severe structural changes are usually irreversible, while the scattering increase associated with SD reversed within a few minutes.

The physical mechanism of the scattering increase remains, for now, unclear. Although the scattering increase does not occur without the sudden hypoxia-induced potential shifts (Müller and Somjen 1998c), it now became apparent that the ΔV of hypoxic SD can proceed without an increase in light scattering. Our most important finding is that, even though the light-scattering increase that coincides with hypoxic SD is CI−-dependent, it is not generated by cell swelling, or by the resulting restriction of extracellular space.

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