Na\textsuperscript{+} and K\textsuperscript{+} Concentrations, Extra- and Intracellular Voltages, and the Effect of TTX in Hypoxic Rat Hippocampal Slices

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Müller, Michael and George G. Somjen. Na\textsuperscript{+} and K\textsuperscript{+} concentrations, extra- and intracellular voltages, and the effect of TTX in hypoxic rat hippocampal slices. J. Neurophysiol. 83: 735–745, 2000. Severe hypoxia causes rapid depolarization of CA1 neurons and glial cells that resembles spreading depression (SD). In brain slices in vitro, the SD-like depolarization and the associated irreversible loss of function can be postponed, but not prevented, by blockade of Na\textsuperscript{+} currents by tetrodotoxin (TTX). To investigate the role of Na\textsuperscript{+} flux, we made recordings from the CA1 region in hippocampal slices in the presence and absence of TTX. We measured membrane changes in single CA1 pyramidal neurons simultaneously with extracellular DC potential ($V_o$) and either extracellular [K\textsuperscript{+}] or [Na\textsuperscript{+}]; alternatively, we simultaneously recorded [Na\textsuperscript{+}]\textsubscript{c}, [K\textsuperscript{+}]\textsubscript{c}, and $V_o$. Confirming previous reports, early during hypoxia, before SD onset, [K\textsuperscript{+}]\textsubscript{c} began to rise, whereas [Na\textsuperscript{+}]\textsubscript{c} still remained normal and $V_o$ showed a slight, gradual, negative shift; neurons first hyperpolarized and then began to gradually depolarize. The SD-like abrupt negative $\Delta V_o$ corresponded to a near complete depolarization of pyramidal neurons and an 89% decrease in input resistance. [K\textsuperscript{+}]\textsubscript{c} increased by 47 mM and [Na\textsuperscript{+}]\textsubscript{c} dropped by 91 mM. Changes in intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations, estimated on the basis of the measured extracellular ion levels and the relative volume fractions of the neuronal, glial, and extracellular compartment, were much more moderate. Because [Na\textsuperscript{+}]\textsubscript{c} dropped more than [K\textsuperscript{+}]\textsubscript{c}, increased, simple exchange of Na\textsuperscript{+} for K\textsuperscript{+} cannot account for these ionic changes. The apparent imbalance of charge could be made up by Cl\textsuperscript{−} influx into neurons paralleling Na\textsuperscript{+} flux and release of Mg\textsuperscript{2+} from cells. The hypoxia-induced changes in interneurons resembled those observed in pyramidal neurons. Astrocytes responded with an initial slow depolarization as [K\textsuperscript{+}]\textsubscript{c} rose. It was followed by a rapid but incomplete depolarization as soon as SD occurred, which could be accounted for by the reduced ratio, [K\textsuperscript{+}]\textsubscript{c}/[Na\textsuperscript{+}]\textsubscript{c}. TTX (1 \mu M) markedly postponed SD, but the SD-related changes in [K\textsuperscript{+}]\textsubscript{c} and [Na\textsuperscript{+}]\textsubscript{c} were only reduced by 23 and 12%, respectively. In TTX-treated pyramidal neurons, the delayed SD-like depolarization took off from a more positive level, but the final depolarized intracellular potential and input resistance were not different from control. We conclude that TTX-sensitive channels mediate only a fraction of the Na\textsuperscript{+} influx, and that some of the K\textsuperscript{+} is released in exchange for Na\textsuperscript{+}. Even though TTX-sensitive Na\textsuperscript{+} currents are not essential for the self-regenerative membrane changes during hypoxic SD, in control solutions their activation may trigger the transition from gradual to rapid depolarization of neurons, thereby synchronizing the SD-like event.

INTRODUCTION

Acute severe hypoxia of forebrain gray matter induces self-regenerating rapid depolarization of neurons that closely resembles spreading depression (Leão 1947). Spreading depression (SD) is characterized by loss of neuronal activity, nearly complete depolarization of neurons and glial cells, and substantial disturbance of the ionic distribution (Grafstein 1956; Marshall 1959). During both normoxic SD and hypoxic SD-like depolarization, the extracellular concentrations of the major inorganic cations and anions are shifted far beyond the physiological range, greatly in excess of the ionic changes associated with tetanic stimulation or seizures (Hansen 1985; Nicholson 1984; Nicholson and Kraig 1981). From the outset, the question has been raised whether SD represents a breakdown of the normal selective permeability of neuron membranes or whether it is produced by the abnormal operation of physiological ion channels. This basic question has not been satisfactorily answered.

SD can be provoked even when action potentials and synapses are blocked by tetrodotoxin (TTX) (Sugaya et al. 1978; Tobiasz and Nicholson 1982). Similarly, hypoxic SD-like depolarization is not usually blocked by TTX, but its onset is delayed considerably and, in a minority of cases, prevented by the drug (Aitken et al. 1991; Xie et al. 1994). Yet several observations suggest that SD is not the consequence of membrane breakdown or the complete loss of membrane resistance (Czéh et al. 1993; Müller and Somjen 1998; Phillips and Nicholson 1979). Moreover, in a previous study (Müller and Somjen 1998), we demonstrated that hypoxic SD can be prevented completely if voltage-sensitive Na\textsuperscript{+} and Ca\textsuperscript{2+} channels as well as AMPA/kainate and N-methyl-D-aspartate (NMDA) glutamate receptors are all blocked. A role for physiological channels also is suggested by the fact that, in current-clamp recordings from CA1 pyramidal neurons, the hypoxic SD-like depolarization is triggered at an apparent threshold potential of approximately $-52$ mV, which is almost identical to the Na\textsuperscript{+} spike threshold of $-53$ mV reported by Dingledine (1983).

These observations reopened the question, what role, if any, is played by voltage-gated Na\textsuperscript{+} channels in the evolution of SD? The main purpose of the present study was to answer this question. To this end, we obtained current-clamp recordings from CA1 pyramidal neurons, interneurons, and glial cells during severe hypoxia while extracellular potential and either [K\textsuperscript{+}]\textsubscript{c} or [Na\textsuperscript{+}]\textsubscript{c} also were monitored. Unlike in previous studies, these parameters were not measured one by one but were monitored simultaneously in the same spot of a given slice. In other trials, we used triple-barreled microelectrodes, simultaneously measuring extracellular potential, [K\textsuperscript{+}]\textsubscript{c} and [Na\textsuperscript{+}]\textsubscript{c} in a single point to resolve the time course and inter-relationship of these parameters. These experiments first were performed in control slices and then repeated after application.
of TTX, revealing the TTX sensitivity of each of the measured variables.

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

METHODS

Preparation

Hippocampal tissue slices were prepared from ether-anesthetized, male Sprague–Dawley rats of 119–265 g body wt (4–7 wk old). After decapitation, the brain was removed rapidly from the skull and placed in chilled artificial cerebrospinal fluid (ACSF) for 1–2 min. The two hemispheres were separated, 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 of the Oslo style and were left undisturbed for ≥90 min. The recording chamber was kept at a temperature of 34.5–35.5°C. It was aerated continuously with 95% O₂-5% CO₂ (400 ml/min), and perfused with oxygenated ACSF (1.5 ml/min). Hypoxia was induced by switching the chamber’s gas supply to 95% N₂-5% CO₂. 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 cm²) 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 NaH₂PO₄, 24 NaHCO₃, 1.2 CaCl₂, 1.2 MgSO₄, and 10 dextrose; aerated with 95% O₂-5% CO₂ to adjust pH to 7.4. TTX (tetrodotoxin, citrate buffered, Calbiochem and Sigma) was prepared Fluka 60031) and backfilled with 150 mM KCl ACSF (1.5 ml/min). Hypoxia was induced by switching the chamber’s fluid, Na⁺ in the calibration solutions was replaced by K⁺ to K⁺-sensitive electrodes and 150, 100, 50, 20, 10, 5, 1, and 0 mM Na⁺ for Na⁺-sensitive electrodes). To maintain constant ionic strength similar to that in interstitial fluid. Na⁺ in the calibration solutions was replaced by K⁺ and vice versa (reciprocal calibration method). Average slopes of the K⁺- and Na⁺-sensitive electrodes were 54.6 ± 2.9 mV/decade K⁺ and 50.8 ± 3.6 mV/decade Na⁺; their detection limits were 0.25 ± 0.23 mM K⁺ (n = 11) and 3.7 ± 0.3 mM Na⁺ (n = 20).

Occasionally we used triple-barreled microelectrodes of the twisted type for simultaneous Na⁺ and K⁺ recordings. A single-barreled capillary (1B150F-4, WPI) was glued to the double-barreled theta-type capillary (GCT 200–10, Clark Electromedical Instruments) using a slow-setting two component epoxy glue (Posy Pro, Power Poxy Adhesives). To achieve maximum adhesion strength, the glue was hardened by placing the capillary tubes in the oven (60°C, 1 h). The capillaries then were pulled on a vertical puller (Narishige PE-2). In a first step, the glass was melted and the capillaries were pulled by only 2–4 mm, simultaneously twisting the attached reference barrel by 180° around the centered theta capillary. After cooling, capillaries were pulled apart in a second pulling step. Both barrels of the theta capillary were silanized by exposure to HDMDS vapor and subsequent baking in the oven. Silanization of the attached reference barrel was prevented by filling it with distilled water. Fillings of the Na⁺- and K⁺-sensitive barrels were the same as for the double-barreled electrodes and the reference barrel contained 150 mM NaCl +10 mM HEPES, pH 7.4. Electrode resistances of the Na⁺-sensitive, K⁺-sensitive, and reference barrels were 200, 130, and 40 MΩ, respectively. Electrode slopes and detection limits were comparable with those of the double-barreled electrodes. There was no noticeable interference between adjacent barrels.

Microelectrodes

Single-barreled glass microelectrodes for extracellular recordings were pulled from thin-walled borosilicate glass (TW150F-4, WPI) using a horizontal puller (P-80/PC, Flaming Brown). They were filled with ACSF, and their tips were broken to a final resistance of 5–10 MΩ. Sharp microelectrodes for current-clamp recordings were made from thick-walled borosilicate glass (1B150F-4, WPI) and filled with 2 M K-Acetate +5 mM KCl +10 mM N-2-hydroxymethylpropazine-N'-2-ethanesulfonic acid (HEPES; Sigma); pH 7.4. Their resistances were 60–80 MΩ. Extracellular Na⁺ and K⁺ concentrations were measured using double-barreled Na⁺- or K⁺-sensitive microelectrodes. They were pulled from theta type capillaries (GCT 200–10, Clark Electromedical Instruments) on the horizontal puller. The designated ion-sensitive barrel was silanized by 60-min exposure to HDMDS vapors (hexamethyldisilazane, 98%, Fluka; vaporized at 40°C) and subsequent baking in the oven (200°C, 2 h). Silanization of the reference barrel was prevented by flushing it with compressed air (1.5 bar).

The tip of the K⁺-sensitive barrel was filled with the valinomycin-based K⁺ ion neutral carrier (Potassium Ionophore I –Cocktail A, Fluka 60031) and backfilled with 150 mM KCl +10 mM HEPES, 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 and 80–110 MΩ, respectively.

The widely used ETH 157 Na⁺ neutral carrier (Sodium Ionophore II Cocktail A, Fluka 71178) was found to be unsuited for extracellular Na⁺ measurements during SD, due to its high K⁺ sensitivity. Because this Na⁺ carrier is only 2.5 times more sensitive to Na⁺ than to K⁺ (log FNa/K = −0.4), the electrode response strongly deviated from linearity in the range where [Na⁺] was expected to be low and [K⁺] high, and the constructed electrodes exhibited an average detection limit of 23 mM Na⁺. We therefore prepared a Na⁺-sensitive cocktail based on the more selective Na⁺ Ionophore VI (Fluka 71739), which is 100 times more sensitive to Na⁺ than to K⁺ (log FNa/K = −2.0). According to Deitmer and Munsch (1995) the Na⁺ ionophore VI, the organic solvent 2-nitrophenyl octyl ether (Fluka 73732) and the lipophilic salt potassium tetraphenylborate (Fluka 72018) were mixed at the weight percent ratio 10.0:89.5:0.5, respectively.

Ion-sensitive electrodes were calibrated before and after each experiment by detecting their response generated in standard solutions (0, 1, 2, 5, 10, 20, 50, and 100 mM K⁺ for K⁺-sensitive electrodes and 150, 100, 50, 20, 10, 5, 1, and 0 mM Na⁺ for Na⁺-sensitive electrodes). To maintain constant ionic strength similar to that in interstitial fluid. Na⁺ in the calibration solutions was replaced by K⁺ and vice versa (reciprocal calibration method). Average slopes of the K⁺- and Na⁺-sensitive electrodes were 54.6 ± 2.9 mV/decade K⁺ and 50.8 ± 3.6 mV/decade Na⁺; their detection limits were 0.25 ± 0.23 mM K⁺ (n = 11) and 3.7 ± 0.3 mM Na⁺ (n = 20).

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Electrical recordings

Ion-sensitive electrode signals were referred to an Ag/AgCl bridge electrode embedded in 2% agar in 3 M KCl. They were recorded by a DC amplifier (constructed locally) and digitized by a TL-1/1 Lab Master acquisition system at sampling rates of 25 Hz ([Na⁺]o,[K⁺]o, measurements only) or 1 kHz (combined current-clamp and [Na⁺]o,[K⁺]o measurements). Because electrodes were calibrated to Na⁺ and/or K⁺ concentrations and the activity coefficient of the measured ion was held constant, changes in [Na⁺]o and [K⁺]o could be calculated directly from the electrode responses using the electrodes’ averaged slope of pre- and postexperiment calibration.

All signal amplitudes were measured between the prehypoxia baseline and the maximal change. Only rapid negative extracellular DC potential changes (∆Vdc) of ≥10 mV amplitude were considered as SD. SD onset was defined as occurrence of the sudden ∆Vdc.

Current-clamp recordings from CA1 pyramidal neurons were performed with an intracellular recording amplifier (Neuro Data, IR-283). Bridge balance and electrode-capacitance compensation were adjusted before insertion of the electrode and continuously controlled during the entire recording. CA1 pyramidal neurons were identified by their location in stratum pyramidale, membrane potential, spontaneous activity, action-potential shape, and input resistance (Morin et al. 1996). Interneurons were identified by their location in st. radiatum close to the pyramidal cell layer, their nonadaptive spike firing in response to depolarizing stimuli, prominent spike-afterhyperpolarizations as well as a pronounced inward rectification during hyperpolarizing current pulses (Morin et al. 1996). Astrocytes were identified by
their location in st. radiatum lining the pyramidal cell layer (D’Ambrosio et al. 1998), very negative membrane potential, low input resistance, and absence of spike discharges in response to depolarizing stimuli. Successful cell impalement was achieved by slowly advancing the electrode into the slice and applying a brief high-frequency oscillating AC signal to the electrode. Cell recovery was facilitated by injecting a hyperpolarizing current for the first few minutes after impalement. Only neurons with a stable membrane potential of at least −55 mV and glial cells with a membrane potential of at least −75 mV were accepted. Neuronal input resistance was determined every 10 s by injecting a hyperpolarizing current of 400-pA amplitude and 200-ms duration. Data were sampled at 1 kHz using the TL-1 Labmaster acquisition system and the Axotape V2 software (Axon Instruments). Input resistances were measured at the steady state level of the voltage deflections and averaged over 10 successive current injections. Changes in input resistance were expressed as a percent of pretreatment value.

** Estimates of intracellular Na\(^+\) and K\(^+\) changes **

On the basis of the measured [Na\(^+\)]\(o\) and [K\(^+\)]\(o\) changes during SD, we calculated the intracellular ion changes. These estimates were based on an averaged interstitial volume fraction (ISVF) of 0.15 (McBain et al. 1990; Pérez Pinzón et al. 1995) and intracellular volume fractions (ICVF) of 0.45 for neurons and 0.40 for glial cells, based on Kuffler and Nicholls (1966) much-quoted figure of glial cells occupying ≥50% of the cellular space. The fraction of glial volume may in hippocampus be <50% (see e.g., Wolff 1966; reviewed by Somjen 1975). Also the ISVF at rest may actually be >15% (Mazel et al. 1998), whereas during SD it shrinks to a much smaller size (Jing et al. 1994; Pérez-Pinzón et al. 1995). For now, the calculations are only intended to show limits; the true intracellular changes are probably within the calculated range. The intracellular ion changes (Δ[X]\(_i\)) were calculated according to the formula

\[
Δ[X] = −Δ[X]_o \times \text{(ISVF/ICVF)}
\]

Calculations were made for the two different assumed conditions that only neurons are subject to Na\(^+\) influx and K\(^+\) release (ICVF = 0.45) or that both neurons and glial cells take up Na\(^+\) and release K\(^+\) to an equal amount during hypoxic SD (ICVF = 0.85).

** Statistics **

The data were obtained from 32 rats, and because most experiments did not last longer than 2 h, up to four slices could be used from each brain. All numerical values are represented as means ± standard deviations. Significance of the observed changes 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 **

**Interrelationship of pyramidal neuron membrane properties, \(V_o\), \([Na^+]_o\), and \([K^+]_o\) **

To investigate the interrelationship of intracellular voltage changes and extracellular ion concentrations during hypoxic SD, we recorded the intracellular potential of CA1 pyramidal neurons and measured \(V_o\) combined with either \([Na^+]_o\) or \([K^+]_o\), in the extracellular space of st. pyramidale close to the impaled neuron. The extracellular ion changes are summarized in Table 1.

The mean resting membrane potential of pyramidal neurons was −62.6 ± 5.0 mV and their input resistance averaged 39.0 ± 7.3 MΩ (n = 22). Oxygen withdrawal almost immediately caused an initial hyperpolarization of 4.3 ± 2.5 mV and a decrease of the input resistance by 37.8 ± 14.8% (Figs. 1 and 2). The initial hyperpolarization abolished spontaneous activity within the first 30 s of hypoxia. At the same time \([K^+]_o\) increased slowly, which suggests release of K\(^+\) from neurons via the activated K\(^+\) channels that generated the hyperpolarization (Hansen et al. 1982) (Fig. 1). After ~1 min, the initial hyperpolarization turned into a gradual depolarization, whereas input resistance remained low and \([K^+]_o\) continued to increase. During this initial phase of hypoxia, \([Na^+]_o\) remained unchanged (Fig. 2).

Within 1.8 ± 0.5 min of hypoxia the intracellular potential \(\left(V_o\right)\) depolarized to −51.6 ± 4.3 mV (n = 22), the apparent threshold potential at which SD started. This apparent SD threshold is almost identical to the Na\(^+\) spike threshold of −53 mV (Dingledine 1983). At the onset of SD \([K^+]_o\) averaged 9.0 ± 3.6 mM (n = 10), which is below the so-called “K\(^+\) ceiling level,” a limit that is not exceeded during tetanic stimulation or even seizures (Heinemann and Lux 1977; Nicholson 1984).

Immediately before SD onset, indicated by the sudden Δ\(V_o\), pyramidal neurons discharged multiple spikes and then their \(V_o\) rapidly rose to −23.2 ± 4.9 mV followed by a more gradual depolarization to a final level of −7.8 ± 5.0 mV. The input resistance decreased by 88.5 ± 11.9% (n = 22). The SD-related sudden negative Δ\(V_o\) averaged −17.8 ± 4.5 mV at the same time \([Na^+]_o\) dropped from its 155 mM baseline to 64.3 ± 13.3 mM (n = 15, Fig. 2) and the already elevated \([K^+]_o\).

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**Table 1. Comparison of hypoxia-induced extracellular ion changes in the dendritic layer (st. radiatum) and the cell layer (st. pyramidale)**

<table>
<thead>
<tr>
<th></th>
<th>Stratum radiatum</th>
<th>St. pyramidale</th>
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</thead>
<tbody>
<tr>
<td>([K^+]_o), mM</td>
<td>Initial</td>
<td>SD</td>
</tr>
<tr>
<td>Stratum radius</td>
<td>7.5 ± 1.5</td>
<td>54.4 ± 12.9</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>St. pyramidale</td>
<td>9.0 ± 3.6</td>
<td>50.9 ± 28.9</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Listed are ionic levels as well as the changes in interstitial volume (ISV) caused by cell swelling during the initial phase of hypoxia, before spreading depression (SD) onset, and at the height of the hypoxia-induced SD. Baseline concentrations are 3.5 mM K\(^+\) and 155 mM Na\(^+\); extracellular space averages 12–20% of brain volume (Mazel et al. 1998; McBain et al. 1990; Pérez-Pinzón et al. 1995). * Extracellular space changes according to M. Müller (unpublished observations). Values are means ± standard deviations. ND, not determined.
Hypoxia-induced membrane changes in interneurons and glial cells

During reoxygenation the membrane potential usually became more negative and the input resistance higher than before hypoxia. Spontaneous action potentials returned within 3–5 min. $[\text{Na}^+]_o$ began to recover, reaching a plateau level of $82.1 \pm 8.7$ mM ($n = 15$). By contrast, neither $V_i$ nor the input resistance recovered before oxygen was readmitted (Figs. 1 and 2).

When reoxygenation was started ~100 s after SD onset, the extracellular ion concentrations immediately started to recover, whereas the recovery of $V_i$ and input resistance was somewhat delayed. In fact, in most pyramidal neurons the depolarization continued briefly before repolarization began (Figs. 1 and 2). During reoxygenation the membrane potential usually became more negative and the input resistance higher than before hypoxia. Spontaneous action potentials returned within 3–5 min. $[\text{Na}^+]_o$ returned to its prehypoxic baseline, whereas $[\text{K}^+]_i$ consistently undershot its 3.5 mM baseline, reaching $1.8 \pm 0.6$ mM ($n = 10$; Fig. 1).

Hypoxia-induced membrane changes in interneurons and glial cells

Occasionally we also recorded from current-clamped interneurons ($n = 11$) and astrocytes ($n = 6$), which were identified according to their location in st. radiatum and their electrophysiological properties (see METHODS). The membrane changes induced by hypoxia in different cell types are compared in Table 2. St. radiatum interneurons had a somewhat more negative membrane potential and a lower input resistance than pyramidal neurons. The hypoxia-induced membrane changes were, however, not noticeably different from those observed in pyramidal neurons. Interneurons also showed an initial hyperpolarization parallel to the increasing $[\text{K}^+]_o$, and they responded with a near complete depolarization and drastically reduced input resistance as soon as the hypoxic $\Delta V_o$ occurred (Fig. 3A).

Glial cells (astrocytes) had the most negative membrane potential (Table 2) and their input resistance was less than a third of that of neurons (see also Leblond and Krnjevic 1989). After oxygen withdrawal, they immediately started to depolarize without the initial hyperpolarization seen in neurons (Fig. 3B). The initial depolarization coincided with the increase in $[\text{K}^+]_o$. As soon as the hypoxic $\Delta V_o$ occurred, glial cells also responded with a sudden depolarization. The amplitude of the SD-like depolarization in glial cells was not different from that in neurons (Table 2), but because glial cells started from more negative resting potentials, they retained a more negative intracellular potential, averaging $-28.2 \pm 2.8$ mV ($n = 6$) at the height of SD (Fig. 3B).
Because of the low glial input resistance, we were not able to measure its changes during hypoxia. Successful glial cell impalement required electrode resistances in excess of 70 MΩ, and current pulses of 1.6–2.0 μA were required to evoke 10- to 15-mV membrane potential deflections. Because electrode resistances were much higher than cell input resistances and tended to change after cell impalement, a stable bridge adjustment could not be maintained. From the few trials it appeared, however, that during hypoxic SD the resistance changes in glial cells are more moderate than those in neurons.

**Estimate of intracellular ion changes and electromotive forces**

Because the intracellular potential, \( V_i \), was recorded in reference to bath (“ground”) potential, the measured change in voltage was the sum of the shifts of the true membrane potential, \( V_m \), plus the extracellular potential shift, \( \Delta V_o \). The difference between \( V_i \) and \( V_m \) becomes maximal at the height of SD. Because the extracellular DC potential does not markedly change during the initial phase of hypoxia, before SD onset, the deviation, however, should be negligible until the rapid depolarization and the negative DC shift start to build up. To estimate \( V_m \) at the height of hypoxic SD, \( \Delta V_i \) must be corrected for \( \Delta V_o \). Figure 4 shows the effect of this correction. During SD \( V_m \) approached but did not quite reach 0 mV. Also the corrected SD-related depolarization has a more “flat top” than in the raw recordings of \( V_i \), indicating that the slow continuing positive shift of \( V_i \) (Figs. 1 and 2) is, in part at least, an artifact caused by contamination by \( \Delta V_o \).

We estimated the changes in intracellular ion concentrations based on the measured extracellular ion changes and the relative volume fractions (see METHODS). We assumed “resting” intracellular concentrations of 10 mM Na\(^+\) (Rose and Ransom 1997a,b) and 140 mM K\(^+\). If during SD Na\(^+\) influx and K\(^+\) release occurs only in neurons, then the calculated [Na\(^+\)] increased to 41.0 ± 5.0 mM (n = 9) and

### Table 2. Hypoxia-induced membrane changes in CA1 pyramidal neurons, interneurons, and glial cells

<table>
<thead>
<tr>
<th></th>
<th>( V_m ), mV</th>
<th>( R_i ), MΩ</th>
<th>Initial ( \Delta V_o ), mV</th>
<th>Initial ( \Delta R_i ), % of Control</th>
<th>SD Threshold, mV</th>
<th>Time to SD Onset, s</th>
<th>( \Delta V ), Total, mV</th>
<th>( \Delta R_i ), Total, % of Control</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyramidal neurons</td>
<td>-62.6 ± 5.0</td>
<td>39.0 ± 7.3</td>
<td>-4.3 ± 2.5</td>
<td>-37.8 ± 14.8</td>
<td>106.4 ± 31.6</td>
<td>54.8 ± 5.3</td>
<td>-88.5 ± 11.9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Interneurons</td>
<td>-66.3 ± 2.7</td>
<td>29.9 ± 11.0</td>
<td>-2.8 ± 1.8</td>
<td>-34.1 ± 13.1</td>
<td>83.8 ± 26.5</td>
<td>57.7 ± 8.3</td>
<td>-82.9 ± 11.9</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Glial cells</td>
<td>-85.0 ± 4.3</td>
<td>ND</td>
<td>No initial hyperpolarization</td>
<td>ND</td>
<td>62.5 ± 7.2</td>
<td>56.8 ± 6.3</td>
<td>ND</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± standard deviations. \( V_m \), membrane potential; \( V_i \), intracellular potential; \( R_i \), input resistance; ND, not determined.

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**FIG. 3.** Hypoxia-induced membrane changes in interneurons and glial cells. A: membrane changes induced by severe hypoxia in interneurons resembled those observed in pyramidal neurons. An initial hyperpolarization was followed by a near complete depolarization paralleled by a massive decrease in input resistance (see also Table 2). B: glial cells, unlike neurons, did not exhibit an initial hyperpolarization but directly responded with a slow depolarization, which eventually turned into a rapid depolarization. At the height of hypoxic SD, however, the glial intracellular potential was still more negative than –25 mV.

**FIG. 4.** Calculated true membrane potential \( (V_m) \) and changes in \([K^+])_i \) (A) and \([Na^+])_i \) (B) based on the recordings shown in Figs. 1 and 2. True membrane potential of pyramidal neurons during SD was calculated by subtracting the extra- from the intracellular potential recordings. Resulting trace shows that the membrane potential is almost nullified as soon as SD occurs. On the basis of the measured \([K^+])_i \) and \([Na^+])_i \) changes, we estimated the changes in \([K^+])_i \) and \([Na^+])_i \), assuming only neurons to be involved (– – –) and neurons and glial cells to be involved equally (—).
\[ [K^+]_o \] dropped to 125.6 ± 5.5 mM \((n = 9)\). These changes would be more moderate if both neurons and glial cells were involved equally (Fig. 4). In this case, \([Na^+]_o\) would increase to 26.4 ± 2.6 mM \((n = 9)\), whereas \([K^+]_o\) would drop to 132.4 ± 2.9 mM \((n = 9)\). If cell swelling is taken into account, then the computed changes of intracellular ion concentrations are even smaller by ~5%.

From the measured change in \([K^+]_o\) and the estimated change in \([K^+]_i\), the \(K^+\) equilibrium potential \((E_K)\) during SD is calculated to shift from \(-97.7\) to \(-27.8\ ±\ 10.8\ mV\), whereas \(E_{Na}\) shifts from \(72.7\) to \(9.8\ ±\ 10.2\ mV\) \((n = 9)\), if we assume that ions flow only into and out of neurons. The assumption that neurons and glial cells participate equally in ion fluxes yields an \(E_K\) of \(-29.2\ ±\ 10.3\ mV\) and an \(E_{Na}\) of 21.4 ± 9.7 mV.

**Simultaneous recordings of \(V_o\), \([Na^+]_o\), and \([K^+]_o\) in control and in TTX-treated slices**

In previous studies, extracellular \(Na^+\) and \(K^+\) usually were recorded separately in different slices. We now used triple-barreled microelectrodes to simultaneously measure \(V_o\), \([Na^+]_o\), and \([K^+]_o\), from one point in st. radiatum in a slice to resolve the magnitude, time course, interrelationship, and TTX sensitivity of these variables. As in st. pyramidale, the onsets of the sharp \([K^+]_o\) increase, the sudden \([Na^+]_o\) drop, and the \(\Delta V_o\) coincided (Fig. 5). The magnitude of the ionic changes in st. radiatum did not differ from those measured in st. pyramidale (Table 1). With the restricted time resolution, inevitable for ion-sensitive microelectrodes, a noticeable time delay between the onsets of the two ion fluxes was not observed. Both ionic changes reached their maximum shortly after SD onset and then began to return toward their normal level, \([Na^+]_o\) more rapidly than \([K^+]_o\). The changes in \([Na^+]_o\) were, however, consistently greater than those in \([K^+]_o\), resulting in an apparent imbalance in cation concentrations averaging 44.3 ± 14.0 mM \((n = 9)\).

After the slices recovered from the first (control) hypoxic SD, we applied 1 \(\mu M\) TTX for \(\geq 30\) min to ensure complete inhibition of TTX-sensitive \(Na^+\) channels, before the second hypoxic SD was induced. In a previous study (Müller and Somjen 1998), we already demonstrated that repetitive induction of hypoxic SD in a single slice kept under control conditions did not induce any significant changes in duration, onset and amplitude of the extracellular DC potential shift (\(\Delta V_o\)) for the first four hypoxic SDs.

TTX markedly delayed the onset of hypoxic SD but only moderately damped the \(\Delta V_o\) amplitude and the changes in \([Na^+]_o\) and \([K^+]_o\) (Fig. 5). After 30-min application of 1 \(\mu M\) TTX, the amplitude of the hypoxic \(\Delta V_o\) decreased to \(78.0\ ±\ 7.9\%\) of control and its onset was delayed by \(143.9\ ±\ 57.0\%\) \((n = 8)\).

TTX reduced the drop in \([Na^+]_o\) during SD only slightly (Fig. 5). In contrast to control slices, \([Na^+]_o\) began to decrease slightly already before SD onset. In the presence of TTX SD was delayed, whereas \([K^+]_o\) continued its slow, pre-SD rise. Therefore at the time of SD onset, \([K^+]_o\) now averaged \(11.6\ ±\ 2.4\ mM\), which is higher than normally but still below the \(K^+\) ceiling level (Heinemann and Lux 1977; Nicholson 1984). The sharp \([K^+]_o\) increase coinciding with SD, however, was damped by \(23.1\ ±\ 20.2\%\), whereas the undershoot of the \(K^+\) baseline after reoxygenation was not significantly affected. The sudden drop in \([Na^+]_o\) coinciding with SD onset decreased by only \(12.2\ ±\ 8.9\%\), and the plateau level decreased by \(18.3\ ±\ 17.0\%\) \((n = 8)\).

**Effects of TTX on the SD-like depolarization in single pyramidal neurons**

As TTX partially reduced the amplitude of the hypoxic \(\Delta V_o\), we asked whether it also reduced the amplitude of the SD-like depolarization in single cells. Because stable impairment could usually not be maintained for >45 min and recordings often were disrupted during reoxygenation, hyp-
oxya under control conditions and in the presence of TTX could not be investigated in the same cells. We therefore compared hypoxia-induced membrane changes during TTX administration to the changes observed in untreated control slices (“control group”). TTX was administered for 20 min before withdrawing oxygen.

As expected, application of 1 μM TTX blocked spontaneous impulse firing. The membrane potentials of TTX-treated pyramidal neurons was 6 mV more negative than that of control cells and their input resistance was 22% lower (see also Fung and Haddad 1997). When oxygen was withdrawn in the presence of TTX, the onset of the SD-like depolarization occurred after 504 ± 131 s (n = 12) of hypoxia, which is >6 min later than in untreated cells (Fig. 6). The initial hyperpolarization was reduced to 51% of the control amplitude, which is probably due to the more negative prehypoxic membrane potential, and the gradual depolarization before SD onset showed a markedly slower time course (Fig. 6, A and B). The final level of the SD-like depolarization and the magnitude of the resistance decrease were not significantly affected by TTX, but the apparent threshold potential of the SD-like depolarization shifted to more positive potentials, averaging now −47.3 ± 5.8 mV (n = 12; Fig. 6C).

DISCUSSION

Changes in \([Na^+]_o\) and \([K^+]_o\)

Simultaneous measurements of extracellular and intracellular changes demonstrated that the onsets of sudden SD-like depolarization of single cells, the drop in \([Na^+]_o\), and the increase in \([K^+]_o\) exactly coincide with the negative deflection of the extracellular DC potential that signals the onset of hypoxic SD. The extracellular ion changes measured during severe hypoxia in the cell body layer (st. pyramidale) and the dendritic layer (st. radiatum) did not show significant differences (Table 1).

Whereas \([K^+]_o\) already increased during the initial, pre-SD phase of hypoxia, \([Na^+]_o\) remained virtually unchanged until the onset of SD. Moderate cell swelling begins during the initial phase of hypoxia restricting extracellular space by ~10% (Jing et al. 1994; Müller and Somjen 1999d), indicating the uptake of solute, most probably NaCl, into neurons and KCl into glial cells. The stability of \([Na^+]_o\) in the face of pre-SD swelling suggests nearly isotonic influx of salt and water with moderate cell swelling and restriction in extracellular space exactly balancing the amount of extracellular Na+ already entering neurons and possibly also glial cells during the initial phase of hypoxia.

The recorded ion concentrations, \([K^+]_o\), which increased to 51–54 mM, and \([Na^+]_o\), which dropped to 61–64 mM (Table 1), are in the ranges previously reported for hypoxic and normoxic SD or ischemia. During anoxia of rat and cat cortex, \([K^+]_o\) increased to levels between 25 and 100 mM and \([Na^+]_o\) decreased to 48–53 mM, whereas ischemia raised \([K^+]_o\) to 75 mM and depressed \([Na^+]_o\) to 48 mM (reviewed by Hansen 1981, 1985). Normoxic spreading depression in rat cerebellum shifted \([K^+]_o\) to 40 mM and \([Na^+]_o\) to 60 mM (Nicholson 1984; Nicholson and Kraig 1981).

After reaching its peak but before reoxygenation, \([Na^+]_o\) already noticeably recovered, whereas \([K^+]_o\) recovered only slightly during hypoxia (Figs. 1, 2, and 5). The partial recovery of extracellular ion concentrations in the absence of oxygen may reflect exchange by diffusion between tissue and bath, but this cannot be the whole explanation because the Na+ diffusion coefficient is smaller than that for K+ \((D_{K,a} = 1.33 \times 10^{-5} \text{ cm}^2/\text{s}, D_K = 1.96 \times 10^{-5} \text{ cm}^2/\text{s})\) (Hille 1992) yet \([Na^+]_o\)
recovered faster than \([K^+]_o\). Instead, these differences might reflect substantially differing kinetics of uptake and/or release of Na\(^+\) and K\(^+\). Because the difference in the time courses of the two ion concentration changes also was observed in the presence of TTX (Fig. 5), inactivation of voltage-gated Na\(^+\) channels is probably not the explanation for this early Na\(^+\) recovery.

Another interesting aspect is the imbalance of extracellular Na\(^+\) and K\(^+\) changes observed at the height of hypoxic SD. In falling from 155 to 64.3 mM, \([Na^+]_o\) dropped by 90.7 mM, whereas in rising from 3.5 to 50.9 mM, \([K^+]_o\) increased by only 47.4 mM, leaving an apparent arithmetic cation deficit of 43.3 mM. Therefore simple exchange of Na\(^+\) for K\(^+\) cannot fully explain the ionic changes during hypoxic SD. Probably most of the difference represents the coupled influx of Na\(^+\) and anions like Cl\(^-\) or HCO\(_3^-\), causing neuronal swelling, with a possible moderate contribution by the release of other cations, especially Mg\(^{2+}\) from cells. Because a large fraction of the total intracellular Mg\(^{2+}\) is bound to ATP, depletion of ATP caused by hypoxia could elevate intracellular Mg\(^{2+}\) activity and thus facilitate Mg\(^{2+}\) release into the extracellular space. In fact, Taylor and coworkers (1999) observed a gradual loss of Mg\(^{2+}\) from cytoplasm, mitochondria, and nucleus during oxygen/glucose deprivation in hippocampal slices.

Estimates of intracellular Na\(^+\) and K\(^+\) changes during hypoxic SD

While hypoxic SD dramatically changed the ionic composition of the extracellular compartment, our estimates indicate much more moderate effects on \([Na^+]_o\) and \([K^+]_o\) (Fig. 4). The difference is due to the smallness of the interstitial volume fraction, which amounts to only 12–20% of the total tissue volume (Mazel et al. 1998; McBain et al. 1990; Pérez-Pinzón et al. 1995). The changes of \([Na^+]_o\) and \([K^+]_o\) were calculated for pyramidal neurons on the basis of the measured extracellular ion concentrations during SD, and assuming two theoretical conditions: that glial cells do not participate in Na\(^+\) uptake and K\(^+\) release during SD and that glial cells and neurons participate equally. The physiological conditions are likely to lie somewhere between these two extremes and will have to be established experimentally by microfluorimetric recordings or the use of ion-sensitive microelectrodes. For now, these calculations are intended only to define the expected limits of intracellular ion changes. According to Friedman and Haddad (1994), \([Na^+]_o\) increased in cultured cortical neurons during anoxia by 27 mM; this fits well into the predicted range.

Membrane responses in single CA1 neurons and glial cells

Neurons as well as glial cells both underwent substantial depolarizations as soon as SD occurred. Yet their membrane responses during the initial phase of hypoxia, before SD onset, clearly differed. Pyramidal neurons and interneurons hyperpolarized by releasing K\(^+\) into the extracellular space. This neuronal K\(^+\) loss apparently reflects activation of K\(_{Ca}\) channels and/or K\(_{ATP}\) channels (Erdemli et al. 1998; Fujimura et al. 1997; Hansen et al. 1982; Leblond and Krnjević 1989), and at least a partial contribution also could be the failure of the Na\(^+\)/K\(^+\) ATPase due to energy shortage. By contrast, glial cells directly responded with a depolarization during the initial phase of hypoxia, as expected from the K\(^+\) sensitivity of glial membrane potential (Kuffler and Nicholls 1966; Somjen 1987).

Although we did not observe noticeable differences in the hypoxic changes induced in pyramidal neurons and interneurons. Congar et al. (1995) reported anoxia to induce a more pronounced outward current in interneurons than in pyramidal neurons. Because of the differing experimental conditions (fully submerged slices, kept at 30–32°C) in the studies of Congar et al. (1995) hypoxic SD did not occur. Instead the anoxic outward currents reported by these authors correspond to the hyperpolarization and the decrease in input resistance we observed during the initial phase of hypoxia before SD onset.

The intracellular potential changes in both, neurons and glial cells exhibited a clearly defined apparent threshold potential at which the slow depolarization turned into the rapid, near complete depolarization (Figs. 1–3). In neurons, this threshold potential is almost identical to the Na\(^+\) spike threshold reported by Dingleedine (1983), and it shifted toward more positive potentials after TTX treatment (Fig. 6). This suggests that, under control conditions, this threshold activation of voltage-gated Na\(^+\) channels triggered SD or at least contributed to the process.

Unclear is, whether activation of glial Na\(^+\) channels contributes to the rapid glial depolarization. Although hippocampal astrocytes do posses voltage-gated Na\(^+\) channels, the K\(^+\) conductance of their membranes is at least fourfold higher than the Na\(^+\) conductance, and their membrane potential is determined mostly by the distribution of K\(^+\) across their membrane (Bordey and Sontheimer 1997; Dennis and Gerschenfeld 1969; Kuffler and Nicholls 1966). The rapid depolarization observed in astrocytes at the onset of SD is therefore probably the result of the elevated \([K^+]_o\) rather than Na\(^+\) influx. The measured increase in \([K^+]_o\) from 3.5 to 50.9 mM is expected to cause a “Nernstian response” of 70.9 mV, at least if \([K^+]_i\) is assumed to remain constant. This calculated potential shift is larger than the 57-mV depolarization observed in glial cells. The difference could be explained by Cl\(^-\) influx into glial cells (Coles et al. 1989). If, however, glial cells would lose K\(^+\) during SD, then their depolarized intracellular potential, −28.2 mV, would be near the calculated \(E_K\). In the few instances that it could be measured, glial input resistance was less dramatically reduced than that of neurons. This agrees with whole cell patch-clamp recordings made by Czéh et al. (1992), whereas in cat cortex, Sugaya and coworkers (1978) found no change in glial input resistance during normoxic SD. These facts point to neurons as the primary generators of hypoxic SD, whereas glial cells appear to play a more passive role.

Glial cells probably protect neurons before SD onset by buffering the excess of \([K^+]_o\) and controlling the ionic composition of the extracellular space (Orkand et al. 1966; Somjen 1987). The importance of glial K\(^+\) buffering was demonstrated by Janigro and coworkers (1997), who observed ictal-like events and epileptiform afterdischarges when glial K\(^+\) uptake was blocked by Cs\(^+\). Eventually, however, the glial buffering capacity is overwhelmed because \([K^+]_o\) continues to rise during hypoxia and glial gap junctions might be closed due to intracellular acidification.

A more than purely passive role of the glial syncytium in the generation and propagation of SD may be indicated by the inhibition of normoxic SD in the presence of the gap-junction
uncoupling agent heptanol (Largo et al. 1997b) as well as the observation that glial cells release glutamate during SD (Basarsky et al. 1999). Heptanol treatment did, however, not affect the generation and propagation of hypoxic SD (Aitken et al. 1998). Also the amount of glutamate released from glial cells is quite small (only 20% of total release) compared with the amount released via Ca\(^{2+}\)-dependent exocytosis (Basarsky et al. 1999). Therefore one might wonder whether glial glutamate release really could indicate a pivotal glial contribution to the generation of SD. Furthermore the efficacy of glutamate antagonists varies among normoxic and hypoxic SD. Although the generation and propagation of normoxic SD can be blocked by glutamate antagonists (Lauritzen and Hansen 1992; Mar- ranes et al. 1988), in the case of hypoxic SD these treatments only caused a postponement of SD onset and a reduction in \(\Delta V_m\) amplitude. Neither could a complete inhibition of hypoxic SD be achieved by combined application of NMDA and non-NMDA inhibitors (Jing et al. 1993; unpublished observations). From these findings, it appears that glial cells may contribute differently to the generation and propagation of normoxic and hypoxic SD, being apparently more important under normoxic than hypoxic conditions.

A predominantly active contribution of glial cells to the generation of SD also is questioned by the ineffectiveness of fluoroacetate and fluorocitrate. Both hypoxic as well as normoxic SD still can be induced after metabolic poisoning of glial cells (Largo et al. 1997a,b; Müller and Somjen 1999c,d). Of course the poisoning actions of these agents are not restricted to glial cells only, but earlier studies showed clearly different time scales in the decline of the neuronal function. Morphological damage occurred first in glial cells, and also the gradual glial depolarization, tissue acidosis, and partial loss of \([K^-]_o\) regulation do suggest an early loss in glial function, whereas neurons are at first mostly unaffected (Largo et al. 1996, 1997a).

**Effects of TTX**

Similarly to earlier studies of both normoxic and hypoxic SD (Aitken et al. 1991; Sugaya et al. 1978; Tobiasz and Nicholson 1982; Xie et al. 1994), application of TTX postponed the onset and depressed the intensity of hypoxic SD but failed to prevent it. The protective effect of TTX (Fung and Haddad 1997; Yamasaki et al. 1991) appears to depend mainly on the delayed onset of SD and on the slower depolarization and decrease in input resistance during the initial, pre-SD phase of hypoxia (Figs. 5 and 6, A and B). Once SD occurred, the extracellular ion changes were only slightly depressed and the final amplitude of the depolarization during SD of pyramidal neurons was not changed at all by TTX (Fig. 6C). As in this study, TTX also failed to prevent \([Na^+]_o\) changes during normoxic SD in rat cerebellum (Tobiasz and Nicholson 1982). It appears therefore that during normoxic as well as hypoxic SD only a small amount (12–18%) of the total \(Na^+\) flux involves TTX-sensitive \(Na^+\) channels.

In single pyramidal neurons, TTX caused a small but consistent positive shift of the SD threshold potential (Fig. 6), which—in combination with the slowed gradual, pre-SD depolarization—is apparently responsible for the marked delay in SD onset. The rapidly depolarizing segment of the \(V_i\) trajectory (the b-c segment in Fig. 6), the final level of \(V_i\), reached during SD, and the massive reduction in input resistance were, however, not changed. Therefore the observed 22% decrease in \(\Delta V_m\) amplitude (Fig. 5) cannot be the result of reduced changes on the single cell level but probably reflects less extracellular current flow due to desynchronized activity in single neurons.

SD appeared more delayed by TTX in the recordings of \(V_i\) from neurons than in the extracellular recordings made with triple-barreled ion-sensitive microelectrodes. This may be a matter of sampling individual units out of a desynchronized population, although localized tissue damage inflicted by the triple-barreled microelectrode, facilitating SD onset, also may have played a role.

**Role of TTX-sensitive Na\(^+\) channels in spreading depression**

Without doubt, the rapid SD-like depolarization of hippocampal neurons implies massive \(Na^+\) influx. This is shown not only by the drop in \([Na^+]_o\) but also in the reported shift of the reversal potential of the ischemic depolarization when the \(Na^+\) concentration in the bath of brain tissue slices was reduced (Tanaka et al. 1997). In dissociated cells and cell cultures, anoxia raised \([Na^+]_o\) (Friedman and Haddad 1994). The mechanism of \(Na^+\) entry during SD is less clear. Voltage-activated “fast” \(Na^+\) channels inactivate within a few milliseconds. They may play a part in triggering SD but are unlikely to contribute much to the SD-related inward current itself. A TTX-sensitive persistent \(Na^+\) current is, however, also present in hippocampal neurons (French et al. 1990). Although its maximum amplitude in isolated cells originally was reported to be quite small, it appears to grow considerably as a consequence of cyanide poisoning and hypoxia (Hammarström and Gage 1998) as well as in elevated \([K^-]_o\) (Somjen, unpublished observations). It therefore may be responsible for a substantial fraction of the SD-like depolarization, but much of the \(Na^+\) appears to enter cells through a TTX-insensitive mechanism. A TTX-insensitive slow \(Na^+\) current has been reported (Hoehn et al. 1993), but this finding is disputed by (Chao and Alzheimer 1995).

Inward flow of \(K^+\) through voltage-gated \(K^+\) channels has to be ruled out as a mechanism driving the self-regenerative depolarization because even at the height of hypoxic SD \(E_K\) was more negative than \(V_m\) and therefore the \(K^+\) driving force was directed outwardly at all times.

The events that cause the depolarization in the presence of TTX therefore remain uncertain for now. In a previous study (Müller and Somjen 1998), we demonstrated that hypoxic SD can be prevented by the combined application of \(Ni^{2+}\), TTX, 6,7-dinitroquinoxaline-2,3-dione, and (±)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid. These observations suggest that activation of glutamate receptors might contribute to the ion fluxes during SD. Even though synaptic function and axonal conduction are blocked during hypoxia in the presence of TTX, release of glutamate and/or aspartate still does occur. Depolarization of synaptic terminals because of increasing \([K^-]_o\) may be sufficient to trigger vesicle fusion and transmitter release. In addition, excitatory amino acids also may be released during SD by two extrasynaptic mechanisms: \(Na^+\)-driven glutamate uptake may reverse due to decreased \(Na^+\) driving force (Attwell et al. 1993) and swelling-activated non-selective anion channels in the membrane of glial cells may allow glutamate release into the extracellular space (Basarsky et al. 1993).
et al. 1999; Kimelberg et al. 1990). The relative contribution of these and other possible Na⁺ pathways has yet to be investigated in more detail.

Concluding remarks

Our present study demonstrates that the massive depolarization of pyramidal neurons, interneurons, and glial cells coincided with the sudden drop in [Na⁺]ᵢ, the increase in [K⁺]ᵢ, and the negative ΔVᵢ. Although neuronal depolarization is characterized by a marked decrease of input resistance and Na⁺ influx, the glial depolarization seems to be largely “passive,” caused by increased [K⁺]ᵢ. Administration of TTX delayed the onset of hypoxic SD but did not prevent it. The final amplitude of the hypoxic SD-like depolarization in single TTX-treated pyramidal neurons did not differ from untreated control slices, and once SD set in, ionic maintenance was not much improved by TTX.

We conclude that TTX-sensitive Na⁺ channels mediate only a small portion of the neuronal Na⁺ influx during SD. In the absence of TTX, activation of voltage-gated Na⁺ channels appears to be responsible for the initiation but not the shaping of the hypoxia-induced SD.

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