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Extracellular Volume Fraction and Diffusion Characteristics During Progressive Ischemia and Terminal Anoxia in the Spinal Cord of the Rat

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Summary: Extracellular space (ECS) volume fraction (α), ECS tortuosity (λ), and nonspecific uptake (k'), three parameters affecting the diffusion of substances in nervous tissue, were studied during ischemia and anoxia in the rat spinal cord gray matter in vivo. Progressive ischemia evoked by exsanguination, as well as anoxia evoked by respiratory or cardiac arrest, produced prominent extracellular K^+ and pH changes closely related to a decrease in blood pressure and amplitude of field potentials. With use of ion-selective microelectrodes, the changes in the diffusion parameters were measured by quantitative analysis of concentration–time profiles of tetramethylammonium (TMA^+) applied by iontophoresis concomitantly with ionic shifts. Under normoxic conditions (in rats with blood pressure of 80–110 mm Hg) diffusion parameters in the dorsal horn gray matter at depth 500–900 μm were as follows: $\alpha = 0.20 \pm 0.019$, $\lambda = 1.62 \pm 0.12$, $k' = 4.6 \pm 2.5 \times 10^{-3}\ s^{-1}$ (mean ± SD, n = 39). Extracellular K^+, pH, and diffusion properties gradually changed during progressive ischemia. As the blood pressure fell to 50–60 mm Hg and field potential amplitude to 20–60%, K^+ rose to 6–12 m*M*, pH_e fell by ~0.05–0.1 pH unit, and volume fraction of the ECS significantly decreased, to $\alpha = 0.16 \pm 0.019$ (n = 22). Even though the tortuosity remained virtually constant, the nonspecific uptake significantly decreased to $k' = 3.4 \pm 1.8 \times 10^{-3}\ s^{-1}$. As the blood pressure fell to 20–30 mm Hg and field potential amplitude to 0–6%, K^+ rose to 60–70 m*M*, pH_e fell by ~0.6–0.8 pH unit, and all three diffusion parameters significantly changed. The ECS volume fraction decreased to $\alpha = 0.05 \pm 0.021$, tortuosity increased to $\lambda = 2.00 \pm 0.24$, and TMA^+ uptake decreased to $k' = 1.5 \pm 1.6 \times 10^{-3}\ s^{-1}$ (n = 12). No further increase in extracellular K^+ or changes in the α were found during and up to 120 min after the death of the animal. However, there was a further significant increase in $\lambda = 2.20 \pm 0.14$ and decrease in $k' = 0.4 \pm 0.3 \times 10^{-3}\ s^{-1}$ (n = 24). The acid shift reached its maximum level at ~5–10 min after respiratory arrest and then the pH_e gradually increased by ~0.2 unit. Full recovery to "normoxic" diffusion parameters was achieved after reinjection of the blood or after an injection of noradrenaline during severe ischemia, if this resulted in a rise in blood pressure above 80 mm Hg and a decrease in extracellular K^+ below 12 m*M*. At ~10 and 30 min after this recovery, the ECS volume fraction significantly increased above "normoxic" values, to $\alpha = 0.25 \pm 0.016$ (n = 7) and $\alpha = 0.30 \pm 0.021$ (n = 6), respectively. The λ and k' were not significantly different from the values found under normoxic conditions. Our data represent the first detailed in vivo measurements of diffusion parameters α, λ, and k' during and after progressive ischemia and anoxia. The observed substantial changes in the diffusion parameters could affect the diffusion and aggravate the accumulation of ions, neurotransmitters, metabolic substances, and drugs used in therapy of nervous diseases and thus contribute to ischemic CNS damage. **Key Words:** Anoxia—Diffusion—Extracellular tortuosity—Extracellular volume fraction—Ischemia—Spinal cord.

Anoxia, and to a lesser degree partial ischemia or hypoxia, has been often shown to be accompanied by excessive ionic shifts in the extracellular space (ECS). Extracellular K^+ increase, Na^+ decrease, Ca^{2+} decrease, and excessive acidosis occur within minutes after a decrease in blood pressure below 80 mm Hg (for reviews see Syková, 1983, 1992; Hansen, 1985). These transmembrane ionic shifts are accompanied by a redistribution of water, and

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Abbreviations used: ECS, extracellular space; ISM, ion-selective microelectrode; TMA^+, tetramethylammonium ion.

the nerve cells, presumably both neurons and glial cells, swell. Shrinkage of the ECS compensates for an increase in the intracellular space volume fraction. A relative and abrupt decrease of the ECS volume by ~50% has been described during anoxia in vivo (Hansen and Olsen, 1980; Hansen, 1985; Korf and Postrema, 1988), suggesting that the diffusion of any substance in ECS is constrained. However, the measurement of the ECS volume fraction and accurate estimation of the movements of substances and the estimation of their concentrations require the study of diffusion parameters in both normal and pathologically changed tissue.

Recent experiments in vitro as well as in vivo demonstrated that the changes in intra- and extracellular volume result in altered diffusion parameters in ECS, which in turn would affect the activity-related accumulation of ions, neuroactive substances, and metabolites in ECS and their movement toward the target cells and access to capillaries (Nicholson and Rice, 1988; McBain et al., 1990; Svoboda and Syková, 1991; Lehmenkühler et al., 1993). In these studies the real-time iontophoretic method, which employs ion-selective microelectrodes (ISMs) to follow the diffusion of extracellular markers applied by iontophoresis, was utilized (Nicholson and Phillips, 1981; Nicholson, 1992). The diffusion in ECS is constrained by two geometrical factors: extracellular volume fraction (α), which is the fraction of the brain volume that is ECS; and extracellular tortuosity factor (λ), which represents the increased path length for diffusion between two points resulting from various barriers, e.g., cellular membranes, glycoproteins, and macromolecules (Nicholson and Phillips, 1981). Besides that, the diffusion between two points can be affected by either specific or nonspecific uptake (k') (Nicholson, 1992).

The diffusion characteristics and ECS volume fraction have been studied in vitro in slices of rat neostriatum during hypoxia (Rice and Nicholson, 1991). This study revealed progressive shrinkage of the ECS to ~50% (to $\alpha = 0.12 \pm 0.04$, mean ± SD), but no significant changes occurred in tortuosity or nonspecific tetramethylammonium ion (TMA^+) uptake during exposure to hypoxic media with continual availability of glucose. The relatively small increase in extracellular K^+ concentration by 7.7 ± 1.2 m*M* in this study, which is in contrast to the large K^+ increases observed during hypoxia in vivo (for review see Syková, 1983, 1992), suggests that only mild hypoxia has been evoked and/or that the changes in vivo may be different. Diffusion parameters during anoxia have not yet been sufficiently studied in vivo. Recently a preliminary study in rat cortex indicated that the α during terminal anoxia decreases to 0.07 ± 0.01 and $\lambda = 1.63 \pm 0.09$ (mean ± SE), while the k' was not evaluated (Lundbaek and Hansen, 1992). Extracellular volume fraction and diffusion characteristics during progressive ischemia and during the recovery phase from ischemia have not yet been studied in vivo.

In the present study, we have evaluated α, λ, nonspecific TMA^+ uptake k', field potentials, rise in extracellular K^+ concentration, and changes in extracellular pH (pH_e) in the rat spinal cord in vivo. Measurements in spinal dorsal horn gray matter in normoxic rats were compared with those during mild ischemia, severe ischemia, terminal anoxia, and early postischemic periods.

MATERIALS AND METHODS

Animal preparation

All animals were female Wistar rats (250–350 g). The rats (n = 52) were anesthetized with pentobarbital (40–60 mg/kg) and given further injections of anesthetic during the experiments (10–20 mg/kg approximately each hour). The trachea and carotid artery were cannulated to allow artificial ventilation and monitoring of systemic arterial blood pressure. A laminectomy was performed between L2 and L6. The animals were artificially ventilated after an intraperitoneal injection of 100 μg of atropine sulfate to reduce tracheal secretion and paralyzed with an intramuscular injection of D-tubocurarine. Arterial blood pressure and heart rate were monitored. In anesthetized rats, the arterial P_aCO_2 was measured in blood samples by ABL4 K and pH/Blood Gas Analyzer (Radiometer) and ranged in normoxic rats from 32 to 38 mm Hg, P_aO_2 was always >120 mm Hg, and pH ranged between 7.35 and 7.45. The animals were mounted in a rigid frame, and a pool filled with paraffin oil (37°C) was made around the spinal cord by fixing the skin flaps to a frame. To minimize respiratory movements at the lumbar level, the chest was suspended free of the underlying surface, using the spinous processes for attachment. The rest of the body and the hind limbs lay on a heating pad. The body temperature was recorded with a rectal probe and maintained at ~37°C.

Induction of gradual ischemia and terminal anoxia

Progressive ischemia was achieved by exsanguination. The blood (1–3 ml) was taken from the cannulated carotid artery by syringe, either 1 ml repeatedly or 2–3 ml at once. This led to a slow decrease in blood pressure, which in some cases ultimately resulted in the animal's death; in others the blood was reinjected into the animals to achieve reoxygenation and the return of blood pressure to normal values. In some experiments, animals received an injection of noradrenaline before death to produce a transient increase in blood pressure. Terminal anoxia was evoked either by exsanguination or by respiratory arrest or by cardiac arrest induced by the administration of ~0.3–1 ml of 0.5 *M* KCl or of saturated $MgCl_2$ via the carotid artery. In all experiments, the diffusion parameters were related to changes in blood pressure and to an increase in extracellular K^+ concentration. In some ex-

periments the field potentials and extracellular pH were measured simultaneously.

Measurements of ionic and ECS volume changes

K^+ activity was recorded with double-barrel K^+-ISMs filled with a liquid ion exchanger (Corning 477317) or with valinomycin ionophore (Fluka 60031) and prepared by a procedure described previously (Kříž et al., 1974). K^+-ISMs were calibrated in 3, 6, 12, 24, 48, and 90 m*M* K^+ with background of either 150, 147, 141, 129, 105, or 63 m*M* NaCl. The same procedure was adopted to prepare the double-barrel pH-sensitive microelectrodes with the liquid Hydrogen Ion Ionophore II-Cocktail A Fluka (Chvátal et al., 1988; Syková and Svoboda, 1990). A reference barrel served also for simultaneous recordings of the field potentials evoked by supramaximal electrical stimulation of the sciatic nerve.

Dynamic changes in the size of the ECS can be studied by means of the iontophoretic administration of ions to which the cell membranes are relatively impermeable. Their concentration in the ECS is in inverse proportion to the size of the ECS. The ion exchanger for K^+ (Corning 477317) is highly sensitive to TMA^+, which, in small concentrations, is not toxic; and only a small fraction of the released ions does leave the ECS (Kříž and Syková, 1981; Nicholson and Phillips, 1981; Nicholson, 1992). TMA^+ was therefore used for testing changes in the size of the ECS. Double-barrel TMA^+-ISMs were prepared by the same procedure as for K^+-ISMs; however, as a backfilling solution, 150 m*M* TMA-Cl was used instead of 0.5 *M* KCl. Reference barrels contained 150 m*M* NaCl. TMA^+-ISMs were calibrated in 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 m*M* TMA^+ with background of either 3, 10, or 70 m*M* KCl and 150, 143, or 80 m*M* NaCl to yield electrode slope and interference. Extracellular K^+ concentration in ischemic tissue was elevated from several millimolar up to ~70 m*M*. However, at a concentration of 0.3 m*M* TMA^+, the electrode slope was equal at both 3 and 70 m*M* KCl background.

With a current of 60–100 nA, TMA^+ was administered for 60–80 s into the agar or ECS with an iontophoretic pipette. The shank of the pipette was bent, before backfilling with 1 *M* TMA^+, so that it could be aligned parallel to the TMA^+-ISM. Electrode arrays of TMA^+-ISM and iontophoretic pipette were made by gluing both electrodes together with dental cement (Fig. 1). The tip of the TMA^+-ISM was 80–200 μm from the tip of the iontophoretic pipette. The TMA^+ was administered at regular intervals (5–10 min), always with the same current. In some experiments (n = 21), 20-nA forward bias current was used throughout.

TMA^+ diffusion curves were at first recorded in 0.3% agar (Difco, U.S.A.) dissolved in a solution of 150 m*M* NaCl, 3 m*M* KCl, and 0.3 m*M* TMA-Cl, in which by definition $\alpha = 1$, $\lambda = 1$, and $k' = 0$ (free-diffusion values). The diffusion curves were analyzed by fitting the data to a solution of Diffusion Eq. 1 using the computer program VOLTORO developed by Ch. Nicholson (unpublished) to yield the TMA^+ diffusion coefficient (*D*) and the iontophoretic electrode transport number (*n*). Diffusion curves were then recorded in the spinal cord at a depth of 500–900 μm, with the tips of the microelectrode array aligned parallel to the long axis of the spinal cord. With knowledge of *n* and *D*, the parameters α, λ, and *k'* can be obtained when experiment is repeated in the spinal cord.

The α, λ, and *k'* values were determined by a computation procedure developed by Nicholson and Phillips (1981). The results of these studies show that if we incorporate factors α and λ into Fick's law, diffusion in the CNS is described fairly satisfactorily. It has been shown that TMA^+ crosses the membranes of leech glial cells and accumulates within (Ballanyi et al., 1990). Recent studies suggest that TMA^+ also enters vertebrate brain cells (see Nicholson, 1992). This can be a source of error, and therefore nonspecific uptake [k' (s^{-1})] was incorporated into the diffusion equation (Nicholson, 1992). Uptake of TMA^+ is proportional to the extracellular concentration and can be a function not only of average permeability of the membranes to TMA^+ but also of a local volume fraction and the density of membranes in the tissue under study. The possibility that TMA^+ may cross the blood–

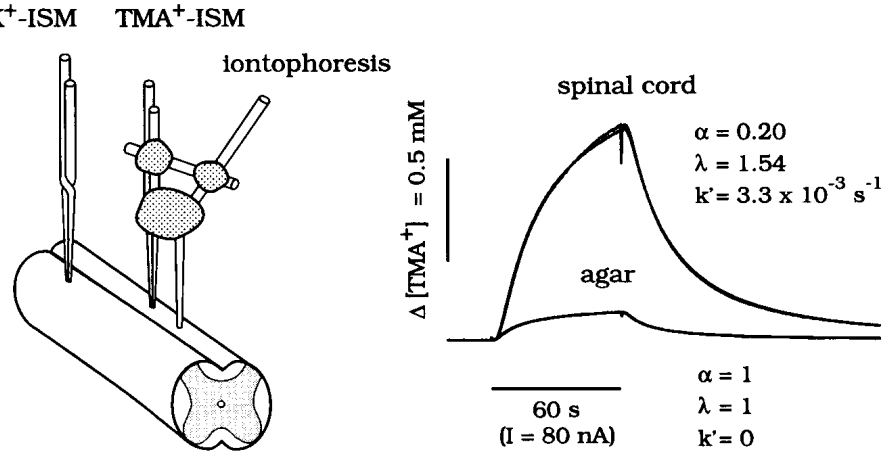

FIG. 1. Method of determining the ECS volume fraction (α), tortuosity (λ), and uptake (*k'*) in spinal cord in vivo. **Left:** Schematic drawing of the experimental arrangement for diffusion measurements. Two microelectrodes, the double-barrel TMA^+-selective microelectrode (TMA^+-ISM) and the micropipette for TMA^+ iontophoresis, were glued together with dental cement at the area of their shanks and with a glass tube bridge connecting them at their upper end to stabilize the intertip distance 80–200 μm. K^+-ISM or pH-ISM was introduced into the spinal cord separately to measure the respective ionic changes. **Right:** Typical diffusion curves obtained in agar gel and in spinal cord. Measurements in agar, where $\alpha = 1$, $\lambda = 1$, and $k' = 0$, enabled the transport number of the iontophoretic electrode to be determined as $n = 0.610$. The iontophoretic current was 80 nA (no bias current was used in this experiment). When the electrode array was lowered 700 μm into the spinal dorsal horn and the same main iontophoretic current applied, the resulting increase in concentration was much larger than that in agar due to the restricted volume fraction ($\alpha = 0.20$) and increased tortuosity ($\lambda = 1.54$). The nonspecific uptake was $k' = 3.3 \times 10^{-3}\ s^{-1}$. The separation between electrode tips was 150 μm. In this figure and all subsequent ones, the TMA^+ concentration scale is linear and the theoretical diffusion curve (Eq. 1) is superimposed on each experimental curve. For abbreviations see the text.

brain barrier is not justified, since uptake values are similar in both in vivo and in vitro preparations. Therefore, assuming that loss of TMA^+ from the ECS is a linear concentration-dependent process, this factor can be incorporated into the diffusion equation.

These three parameters were extracted by a nonlinear curve-fitting simplex algorithm operating on the diffusion curve described by Eq. 1, which represents the behavior of TMA^+, assuming that it spreads out with spherical symmetry when the iontophoresis current is applied for duration S. In this equation C is the concentration of the ion at time t and distance r away. The expression governing the diffusion in brain tissue is

$C = G(t)$ $\quad t < S$, for the rising phase of the curve
$C = G(t) - G(t - S)$ $\quad t > S$, for the falling phase of the curve

The function $G(u)$ is evaluated by substituting t or $(t - S)$ for u in Eq. 1 (Nicholson and Phillips, 1981; Nicholson, 1992; Lehmenkühler et al., 1993):

$$G(u) = \frac{Q\lambda^2}{8\pi D\alpha r}\left\{\exp\left[r\lambda\left(\frac{k'}{D}\right)^{1/2}\right]\operatorname{erfc}\left[\frac{r\lambda}{2(Du)^{1/2}} + (k'u)^{1/2}\right] + \exp\left[-r\lambda\left(\frac{k'}{D}\right)^{1/2}\right]\operatorname{erfc}\left[\frac{r\lambda}{2(Du)^{1/2}} - (k'u)^{1/2}\right]\right\} \quad (1)$$

The quantity of TMA^+ delivered to the tissue per second is $Q = In/zF$, where I is the step increase in current applied to the iontophoresis electrode, n is the transport number, z is the number of charges associated with the substance iontophoresed (+1 here), and F is Faraday's electrochemical equivalent. The function erfc is the complementary error function.

Statistical analysis

Results of the experiments were expressed as means ± SD. Statistical analysis of the differences between groups was evaluated by a one-way analysis of variance. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Diffusion parameters during normoxia

After the TMA^+ diffusion coefficient (D) and the transport number for the iontophoresis pipette (n) were determined in 0.3% agar (for details see Material and Methods), the microelectrode array was inserted from the dorsal spinal surface into the spinal dorsal horn at depths between 500 and 900 μm, where diffusion measurements were made. Another K^+-ISM and/or pH-ISM was inserted into the same depth of the spinal cord, either ~5–10 mm more rostrally or in the contralateral side, to measure changes in extracellular K^+ concentration or pH_e (Fig. 1). Stable diffusion curves were recorded in rats under normoxic conditions (see Material and Methods), characterized by regular heart rate of 380 beats/min and blood pressure between 80 and 110 mm Hg (the normal blood pressure of the rat with body suspended in rigid frame; in a freely moving rat, this represents blood pressure of 130–150 mm Hg). The normoxic animals had, in the spinal dorsal horn, a resting K^+ level of 3.2–3.5 mM and pH_e of 7.1–7.2 due to ongoing spontaneous activity (see also Svoboda et al., 1988; Syková and Svoboda, 1990).

A typical TMA^+ diffusion curve recorded in agar and a spinal cord under normoxic conditions is illustrated in Fig. 1. Superimposed on each experimental curve is the theoretical curve, derived from Eq. 1, using the extracted parameters α, λ, and k' together with the other parameters defining the experiment. A good correlation between experimental and theoretical curves is the test of the quality of the curve-fitting procedure and of the extracted diffusion parameters. Diffusion parameters in the dorsal horn gray matter at depth 500–900 μm were under normoxic conditions as follows: $\alpha = 0.20 \pm 0.019$, $\lambda = 1.62 \pm 0.12$, $k' = 4.6 \pm 2.5 \times 10^{-3}\ s^{-1}$; $n = 39$ (Table 1). No significant differences in α, λ, or k' have been found at a depth from 500 to 900 μm.

Diffusion parameters and ionic changes during and after progressive ischemia

Extracellular K^+, pH, and diffusion properties in the spinal dorsal horn of the rat in vivo were studied during progressive ischemia evoked by gradual exsanguination, i.e., by taking repeatedly 1–2 ml of blood from the carotid artery. As the blood pressure gradually fell to 50–60 mm Hg, field potential amplitude decreased to 20–60% ($n = 16$), K^+ gradually rose to 6–12 mM, and pH_e fell by ~0.05–0.1 pH unit; also the volume fraction of the ECS significantly decreased, to $\alpha = 0.16 \pm 0.019$ ($n = 22$). While the tortuosity had not yet changed, $\lambda = 1.62 \pm 0.14$, the nonspecific uptake had already significantly decreased: $k' = 3.4 \pm 1.8 \times 10^{-3}\ s^{-1}$ (Table 1). In Fig. 2 the increase in amplitude of TMA^+ diffusion curves during ischemia is presented as linear concentration changes with baselines superimposed and fitted to a solution of Eq. 1. The largest curve corresponds to the smallest α.

As the blood pressure fell to 20–30 mm Hg, the field potential amplitude decreased to 0–6% ($n = 21$), K^+ rose to 60–70 mM, and pH_e fell by ~0.6–0.8 pH unit (Table 1; Figs. 3 and 5). All three diffusion parameters significantly changed. The ECS volume fraction decreased to $\alpha = 0.05 \pm 0.021$, tortuosity increased to $\lambda = 2.00 \pm 0.24$, and TMA^+ uptake decreased to $k' = 1.5 \pm 1.6 \times 10^{-3}\ s^{-1}$ ($n = 12$). Typical TMA^+ diffusion curves are shown in Figs. 2 and 4. We did not find any differences in the

TABLE 1. *ECS diffusion parameters as function of ischemia/anoxia–evoked decrease in blood pressure and increase in extracellular K^+ level ($[K^+]_e$) and changes at 10 and 30 min after recovery from ischemia evoked by reinjection of blood*

Blood pressure (mm Hg)	$[K^+]_e$ (m*M*)	α	λ	*k'* (10^{-3} s^{-1})	n
>80	3–4	0.20 ± 0.019	1.62 ± 0.12	4.6 ± 2.5	39
50–60	6–12	0.16 ± 0.019*	1.62 ± 0.14	3.4 ± 1.8*	22
20–30	60–70	0.05 ± 0.021*	2.00 ± 0.24*	1.5 ± 1.6*	12
0	60–70	0.07 ± 0.014	2.20 ± 0.14*	0.4 ± 0.3*	24
Postischemic changes					
>80 (10 min)	3–4	0.25 ± 0.016[a]	1.57 ± 0.18	5.8 ± 1.8	7
>80 (30 min)	3–4	0.30 ± 0.021[a]	1.63 ± 0.14	4.4 ± 2.9	6

α is ECS volume fraction, λ is ECS tortuosity, *k'* is nonspecific TMA^+ uptake, and n is no. of animals. For other abbreviations and further details, see the text.
*, [a] Significant differences.

changes in diffusion parameters during progressive ischemia evoked by exsanguination and hypoxia evoked by respiratory arrest (see Figs. 2, 4, and 6).

Full and quick recovery to "normoxic" diffusion parameters was found after reinjecting the blood or after an injection of noradrenaline, if this resulted in a rise in blood pressure above 80 mm Hg and a decrease in extracellular K^+ below 12 m*M* (Fig. 3). At 10 min after the recovery achieved by reinjecting the blood, the ECS volume fraction significantly increased above "normoxic" values, to $\alpha = 0.25 \pm 0.016$ (n = 7) (see Table 1). About 30 min after ischemia, the ECS volume fraction increase was even larger: $\alpha = 0.30 \pm 0.021$ (n = 6) (Table 1; Fig. 4). At 10 as well as 30 min after ischemia, the λ and *k'* were not significantly different from normoxic values. A typical time course of blood pressure decrease during progressive ischemia and its recovery after injection of noradrenaline are shown in Fig. 3, concomitantly with the simultaneously recorded changes in $[K^+]_e$, field potentials, $[TMA^+]_e$ baseline, and TMA^+ diffusion curves. In all experiments, the recorded parameters—blood pressure, K^+, pH_e, field potential amplitude, and ECS diffusion—changed concomitantly.

Diffusion parameters and ionic changes evoked by terminal anoxia

Terminal anoxia, which either developed gradually from ischemia evoked by exsanguination or resulted from relatively slow heart failure due to respiratory arrest (5–10 min) or from fast heart failure after intraarterial KCl or $MgCl_2$ injection (1 min), was accompanied by immediate changes in the dif-

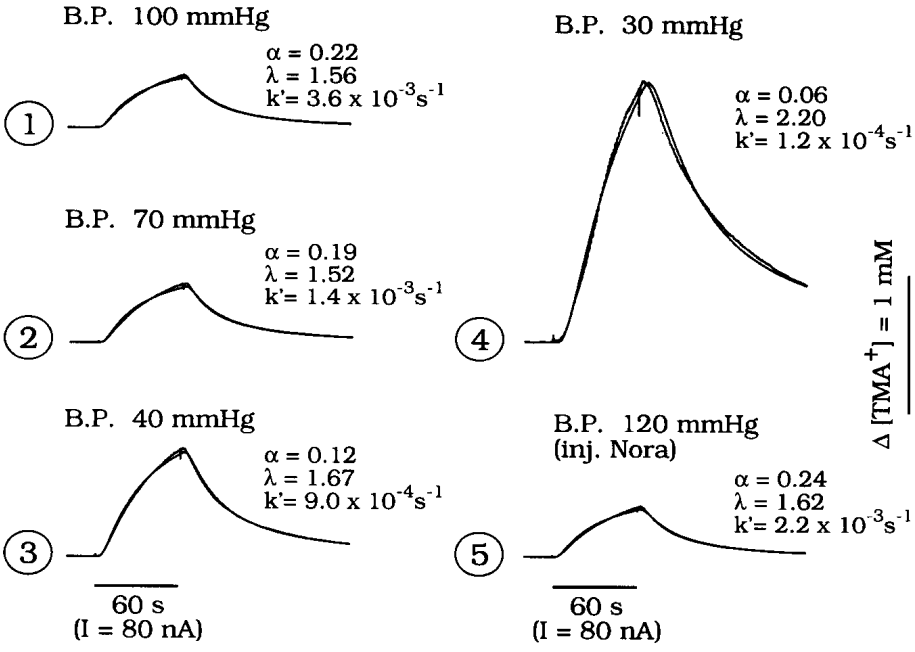

FIG. 2. Tetramethylammonium ion (TMA^+) diffusion curves obtained in the rat spinal cord in vivo in the dorsal horn of segment L4 at a depth of 600 μm from the dorsal surface. Diffusion parameters: Volume fraction (α), tortuosity (λ), and nonspecific cellular uptake (*k'*) were determined with the use of the same electrode array with spacing between the TMA^+-sensitive microelectrode and iontophoresis pipette of 170 μm and iontophoresis transport number *n* = 0.295. The main iontophoretic current was 80 nA (no bias current was used in this experiment). **1:** Diffusion curve was first recorded in normoxic conditions in rat with blood pressure (BP) of 100 mm Hg. Progressive ischemia was evoked by removal of 2 ml of blood from carotid artery. **2–4:** Diffusion curves recorded in animals at BP of 70, 40, and 30 mm Hg. **5:** Diffusion curve obtained 20 min after injection of noradrenaline, which resulted in increase in BP to 120 mm Hg. Note that the increase in curve amplitude reflects the declining volume fraction during ischemia. The values of α, λ, and *k'* are shown with each record. Diffusion curves are from the same rat as in Fig. 3, where the BP changes, increase in extracellular K^+ concentration, field potential changes, and increase in TMA^+ baseline are shown.

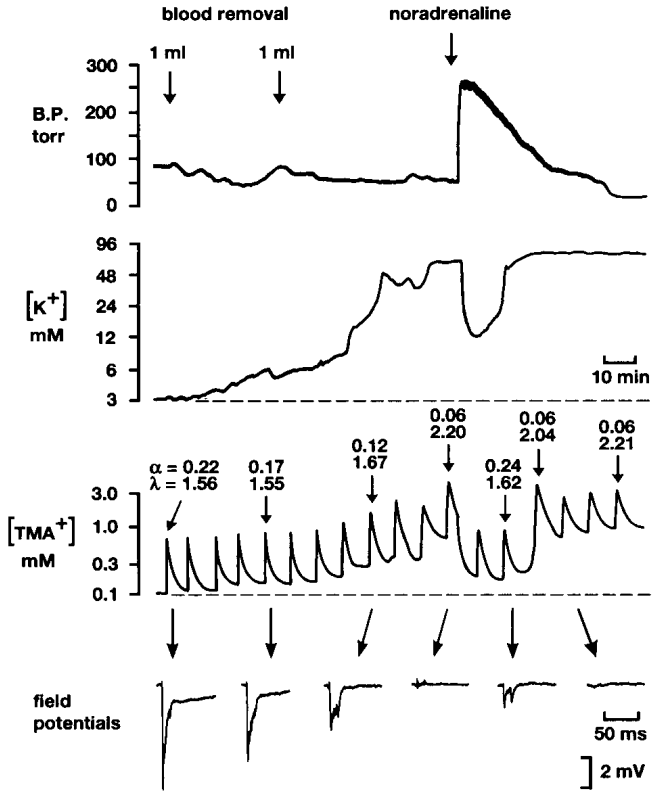

FIG. 3. Simultaneous recordings of the changes in blood pressure (BP), extracellular K^+ concentration, TMA^+ diffusion curves, and field potentials obtained in the rat spinal cord during progressive ischemia evoked by removal of the arterial blood (removal of each 1 ml is marked by an arrow). Diffusion curves are superimposed on the increasing TMA^+ baseline due to the ECS shrinkage. The values of α decreased while λ increased concomitantly with the fall in BP and rise in extracellular K^+. Prior to the animal's death, the transient increase in BP, decrease in extracellular K^+ level, and recovery of diffusion parameters are evoked by an injection of noradrenaline. The values of α and λ are shown above some records. The data are from the same experiment as the diffusion curves in Fig. 2 (see legend to Fig. 2 for further details). For abbreviations see the text.

fusion parameters. At 5–10 min after heart failure, $\alpha = 0.07 \pm 0.014$ (n = 24) was not significantly different from the ECS volume fraction in animals with severe ischemia or hypoxia, i.e., animals with blood pressure of 20–30 mm Hg. However, two other diffusion parameters of spinal cord tissue did change significantly (Table 1). The λ increased to 2.20 ± 0.14, and uptake decreased further to $k' = 0.4 \pm 0.3 \times 10^{-3}\ s^{-1}$ (n = 24). Figures 5 and 6 show typical experiments in which blood pressure dropped due to respiratory arrest, extracellular K^+ level rose to 70 m*M*, and pH_e decreased by ~0.6 pH unit. No further increase in K^+ level or significant changes in the diffusion parameters were found up to 120 min after the death of the animal. Only pH_e reached its highest acidotic shift at ~5–10 min after respiratory arrest or heart failure and then gradually increased by ~0.2 pH unit (Fig. 5) (see also Syková and Svoboda, 1990).

Typically the quality of the curve fitting of the TMA^+ diffusion curves recorded in animals with severe hypoxia or terminal anoxia was not as good as in normoxic animals, as is depicted in Figs. 2 and 6. The curve fitting during anoxia was usually better in experiments in which we did not use bias current, and therefore, there was a relatively lower TMA^+ baseline. This was especially true in experiments in which the TMA^+ diffusion curves were recorded repeatedly during severe hypoxia or in dead animals in which the TMA^+ baseline gradually increased after each application, suggesting that the TMA^+ dissipated from the ECS at a slower rate, apparently because of the large changes in diffusion parameters, i.e., extremely small volume fraction α, large tortuosity λ, and decreased nonspecific uptake k'. In some curves, improvement in the curve fitting had been achieved when the rising phase and falling phase of the diffusion curve were fitted with different values of k'. One of the possible explanations could be changes in TMA^+ baseline, which often occur during anoxia (Fig. 3). Even if this hints at a possible need for some refinement of the theory, the α and λ values obtained in our experiments cannot be significantly distorted by the observed small differences between the experimental and theoretical curves.

DISCUSSION

Diffusion parameters during normoxia

Recently, the values of α and λ under normoxic conditions in vivo were determined in the spinal dorsal horn of the rat (Svoboda and Syková, 1991). In the present study, the nonspecific TMA^+ uptake (k') (Nicholson and Phillips, 1981; Nicholson, 1992), which represents the linear concentration-dependent loss of TMA^+ from the ECS to the intracellular space, blood, or CSF, was also determined. In our original study with the TMA^+ method, we were not able to incorporate k' into our curve-fitting procedure, and we found that many of the diffusion curves could not be fitted (Svoboda and Syková, 1991), particularly their falling phase. In the present study, we incorporated linear uptake (k') and were able to fit almost all curves. Figure 7 shows how the fitting of a typical diffusion curve is affected by k'. The rising and falling phases of the curve (same as curve no. 1 from Fig. 6) were fitted with uptake (a) and without uptake (b) and only the rising phase without uptake (c). It is evident that the curve cannot be fitted and the correct values of λ extracted without incorporation of linear uptake k' (for further details see Nicholson, 1992).

The normoxic α, λ, and k' values in spinal cord in

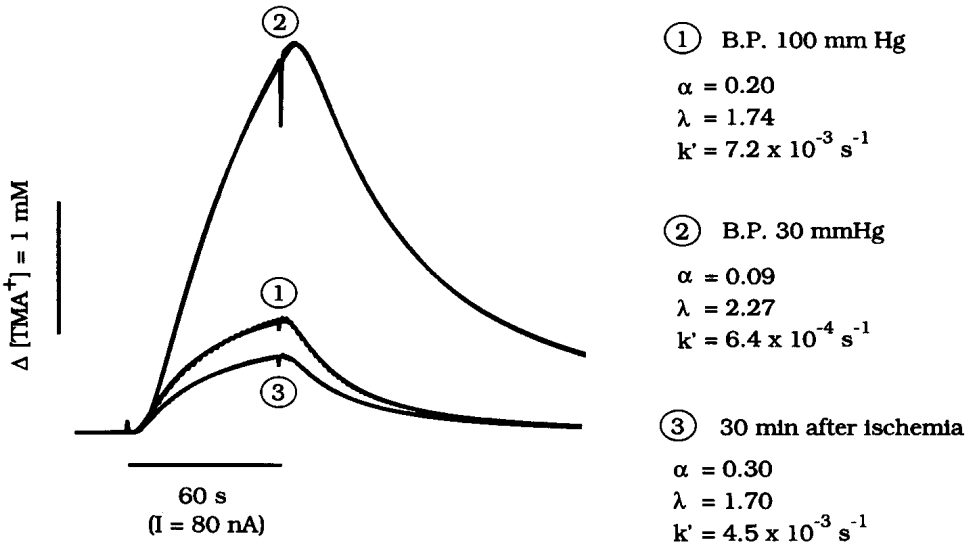

FIG. 4. Tetramethylammonium ion (TMA^+) diffusion curves obtained in the rat spinal cord in vivo in the dorsal horn of segment L4 at a depth of 600 μm from the dorsal surface. Diffusion parameters: Volume fraction (α), tortuosity (λ), and nonspecific cellular uptake (k') were determined with the use of the same electrode array with spacing between the TMA^+-sensitive microelectrode and iontophoresis pipette of 160 μm and iontophoresis transport number n = 0.406. The main iontophoretic current was 80 nA; no bias current was used in this experiment. **1:** Diffusion curve was first recorded in normoxic conditions in rat with blood pressure (BP) of 100 mm Hg. The ischemia was evoked by blood removal. **2:** Diffusion curve recorded when BP fell to 30 mm Hg. **3:** Diffusion curve obtained 30 min after reinjection of blood and return of BP to 90–100 mm Hg. Note that the increase in diffusion curve amplitude reflects the declining volume fraction during anoxia, while its decrease at 30 min after ischemia points to an increase of the volume fraction. The values of α, λ, and k' are shown with each record.

vivo were not significantly different from those found in the rat cortex (Lehmenkühler et al., 1992, 1993; Svoboda et al., 1992). The value of k' was found to be only two to three times ($1.0–1.3 \times 10^{-2}$ s^{-1}) higher in rat striatal slices (Rice and Nicholson, 1991) or in hippocampal slices (McBain et al., 1990) than in cortex or spinal cord in vivo, which may reflect some loss of TMA^+ into the superfusion medium in preparations in vitro. Each of the parameters α, λ, and k' within the dorsal horn gray matter remained the same in various layers (see also Svoboda and Syková, 1991). The α values were, however, significantly different in various cortical layers (Lehmenkühler et al., 1993); e.g., in layers II and III, the mean α values were 0.19 and 0.20, respectively, while in layers V and VI, the mean α values were 0.22 and 0.23. In striatal slices α was 0.21 (range 0.18–0.24) (Rice and Nicholson, 1991). Many brain regions appear to be homogeneous and isotropic with α of ~0.20 and λ of ~1.5–1.6. So far

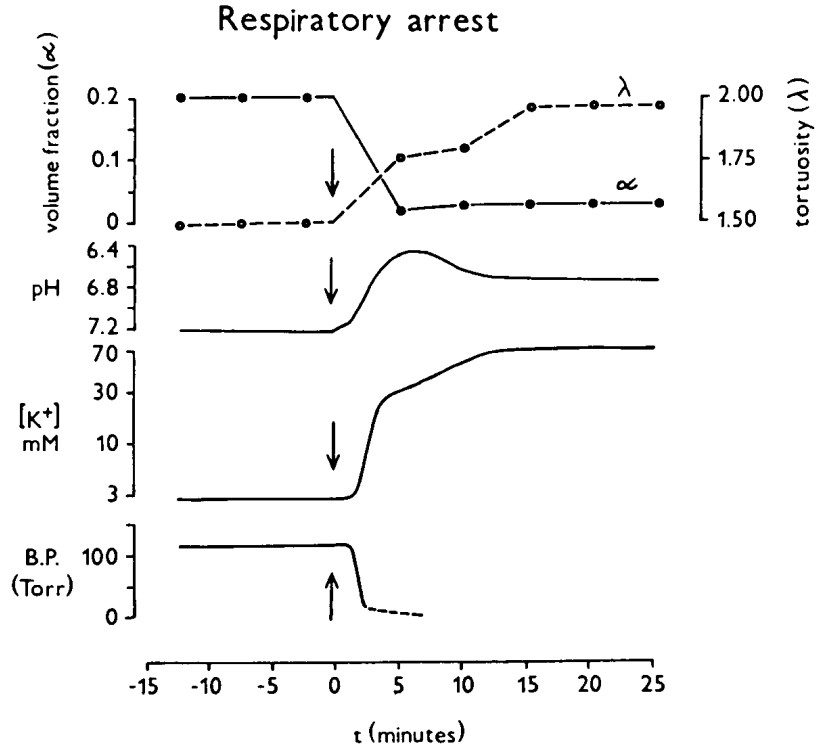

FIG. 5. Decrease in extracellular space volume fraction (α) and increase in tortuosity (λ), increase of extracellular K^+ concentration, and decrease of extracellular pH in the L4 spinal segment at a depth of 600 μm as recorded after respiratory arrest. BP, concomitantly recorded changes in blood pressure.

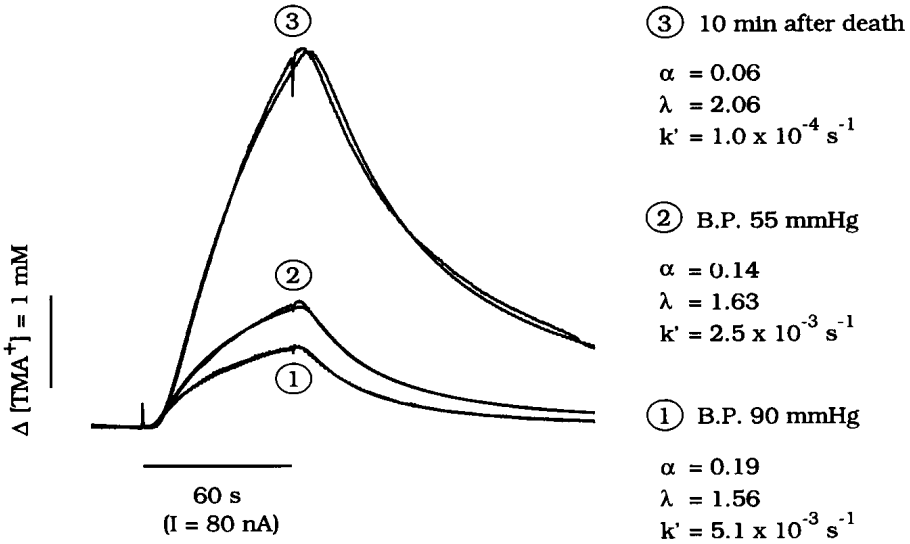

FIG. 6. Tetramethylammonium ion (TMA^+) diffusion curves obtained in the rat spinal cord in vivo in the dorsal horn of segment L4 at a depth of 900 μm from the dorsal surface. Diffusion parameters: Volume fraction (α), tortuosity (λ), and nonspecific cellular uptake (k') were determined with the use of the same electrode array with spacing between the TMA^+-sensitive microelectrode and iontophoresis pipette of 175 μm and iontophoresis transport number n = 0.525. The main iontophoretic current was 80 nA (no bias current was used in this experiment). **1:** Diffusion curve was first recorded in normoxic conditions in rat with blood pressure (BP) of 90 mm Hg. The anoxia was evoked by respiratory arrest. **2:** Diffusion curve recorded during hypoxia when BP fell to 55 mm Hg. **3:** Diffusion curve obtained 10 min after animal's death. Note that the increase in diffusion curve amplitude reflects the declining volume fraction during anoxia. The values of α, λ, and k' are shown with each record.

the only exceptions are the hippocampus and the turtle cerebellum in which local heterogeneity in α and λ was described (McBain et al., 1990; Rice et al., 1993). It remains to be shown, however, whether the local inhomogeneity in α and λ due to structural anisotropy exists in some other regions of the brain or spinal cord.

Progressive ischemia and terminal anoxia

Our data represent the first in vivo measurements of the changes in the diffusion parameters α, λ, and k' during and after progressive ischemia. To date, only the parameters α and λ were reported during terminal anoxia in a preliminary study in the rat cortex (Lundbaek and Hansen, 1992). These authors reported a similar decrease of α values during terminal anoxia as we found in the spinal cord; however, the λ values were significantly lower. Apart from this being due to the fact that they studied brain cortex, it is possible that their technique of analysis was not adequate. Since they did not include linear uptake k' in their fitting procedure, this could explain their lower value of λ during ischemia (see Fig. 7). Furthermore, our study shows that to evaluate the changes in diffusion parameters during ischemia, correlation should be made with at least some of the other parameters, e.g., with extracellular increase in K^+ concentration, changes in pH_e, decrease in blood pressure, and depression of neuronal activity or of the field potentials.

The ECS volume fraction changes of as much as 75% during severe hypoxia and terminal anoxia were greater than the relative ECS volume changes (40–50%) previously determined by the same or

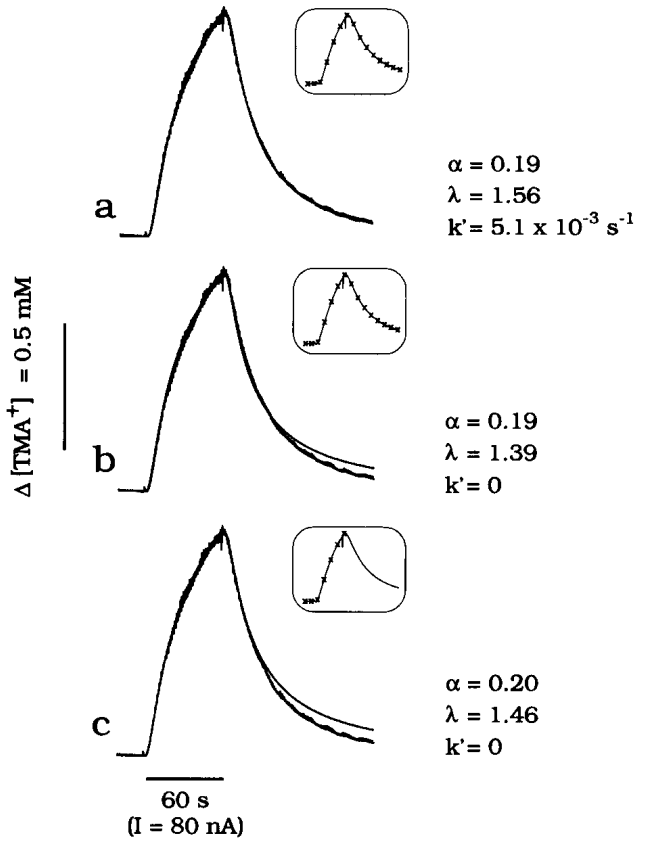

FIG. 7. Fitting of the tetramethylammonium ion (TMA^+) diffusion curve in spinal dorsal horn with and without linear nonspecific uptake k'. **a:** Best correlation between experimental and theoretical curve was obtained when both the rising and the falling phases of the curve were fitted (illustrated in insets) with typical uptake $k' = 5.1 \times 10^{-3}\ s^{-1}$. **b:** Fitting with no uptake ($k' = 0$). **c:** Fitting of the rising phase only, with no uptake ($k' = 0$). The extracted values of α and λ are shown with each record. The data are from the same experiment as the diffusion curve 1 in Fig. 6.

other methods (Hansen and Olsen, 1980; Rice and Nicholson, 1991). The decrease in ECS volume fraction found in our experiments was also greater than the shrinkage of the ECS found during spreading depression or seizures, where the increase in extracellular K^+ is comparable with that observed during severe ischemia (Van Harreveld, 1972; Lipton, 1973; Phillips and Nicholson, 1979). Shrinkage of the ECS after circulatory arrest has already been proposed from measurements of the tissue impedance (Bureš and Burešová, 1957; Van Harreveld, 1966, 1972); this method also recently revealed rapid shrinkage of the rat striatal ECS after ischemia (Korf and Postrema, 1988). Although the impedance method is able to follow the dynamic changes soon after ischemia when the ionic changes begin, it is, however, not able to reveal the absolute values of the ECS volume fraction and changes in other ECS diffusion parameters.

The changes in ECS volume fraction and ECS diffusion parameters that were determined in in vitro slices of rat neostriatum, where α fell from an average value of 0.21 to 0.13 while the tortuosity and k' were unchanged (Rice and Nicholson, 1991), correspond to the changes in spinal cord in vivo during mild ischemia, when blood pressure fell to 50–60 mm Hg. In hypoxic slices of rat neostriatum perfused with the solution bubbled with 95% N_2/5% CO_2, the glucose supply was normal and extracellular K^+ increased only by 2–5 m*M*. This apparently corresponded to a very mild hypoxia during which λ and k' may not yet have changed. In vivo, however, the k' had already decreased during mild ischemia, which might be specific for tissue in vivo, where TMA^+ baseline during ischemia might be higher than in tissue slices perfused with artificial solution. An observed decrease in k' may also be a result of changes in membrane permeability for TMA^+ or a gradual increase in TMA^+ concentration in cells. Even when the uptake is low in both normoxic and anoxic conditions, the TMA^+, especially when its concentrations increase over a sufficiently long time, may be taken into the cells, particularly glia (Ballanyi et al., 1990). A 10-fold smaller k' was found during terminal anoxia. This may be due to the fact that the intracellular TMA^+ concentration increased due to high TMA^+ baseline in the more narrow ECS. We may therefore assume that in vivo, during the excessive extracellular accumulation of TMA^+, its cellular uptake is decreased due to an increased level of TMA^+ within the cells.

The concept of tortuosity was recently described in detail by Nicholson (1992). An increase in tortuosity required severe ischemia (see Table 1), which resulted in ECS shrinkage by >50% as well as in a loss of extracellular K^+ and pH homeostasis. This increase in λ can be explained by the fact that the extracellular matrix becomes more concentrated. It may account for the large change in the geometry of the ECS and shows that the diffusion distance for TMA^+ and for other compounds is increased by the convoluted pathways.

The cellular swelling during cerebral ischemia is mainly a consequence of massive ionic fluxes across the cellular membranes accompanied by movement of water and develops concomitantly with ionic shifts (McKnight and Leaf, 1977; Hansen and Olsen, 1980; Hansen, 1985). Glial cells swell during exposure to elevated K^+, changes of Pco_2, acidosis, nonisotonic media, and excess of neurotransmitters (for review see Walz, 1989; Kempski et al., 1992). The swelling occurs quickly after the interruption of the energy supply, but never before a rise in K^+ and changes in pH. This is in agreement with the hypothesis that besides excitotoxins (e.g., glutamate), acidosis and elevated extracellular K^+ are most likely mediators of glial cell swelling and, consequently, of changes in ECS diffusion parameters (see Walz, 1989; Kempski et al., 1992; Syková, 1992). Indeed, Ballanyi et al. (1990) showed that leech neuropil glial cells swell reversibly during an increase in K^+ concentration in the perfusion solution. Swelling observed at 9, 15, 21, and 40 m*M* K^+ was by 7.5, 14, 18.5, and 50%. However, the neurons could also have contributed to cell swelling and ECS shrinkage during anoxia (Van Harreveld, 1969, 1972). As shown in Fig. 3, the K^+ elevation to 12–70 m*M* that was found during ischemia (see also Syková and Svoboda, 1990) correlates with the ECS volume fraction decrease and tortuosity increase and may well be responsible for the observed ECS shrinkage observed in our study. In fact, there was no further increase in K^+ concentration after the animal's death, and we have also found no further decrease in ECS volume fraction.

It is known that long-lasting ischemia, when followed by recirculation, gives rise to edema formation, which has a "cytotoxic" (early stages) as well as a "vasogenic" (later stages) origin (Klatzo, 1967; Katzman et al., 1977; Siesjö, 1985). However, the postischemic changes in ECS diffusion parameters and their time course have not been studied so far. In this study, we observed after ischemia a quick recovery of the ECS volume, tortuosity, and k' to "normoxic" values, followed within 10 and 30 min by an increase in the ECS volume (see Figs. 2–4). This points either to the early postischemic formation of spinal cord edema, e.g., due to elevated tissue osmolarity (Gisselson et al., 1992), or to post-

ischemic shrinkage of nerve cells. Thus far, the postischemic changes in diffusion parameters have been studied only in relatively early postischemic periods (at 10 and 30 min after ischemia), and further analysis of the postischemic diffusion parameters, ionic changes, and neuronal activity is underway in our laboratory.

Implication and conclusions

In our study, we found no differences in changes of diffusion parameters during ischemia (reduction of blood flow to spinal cord) or during hypoxia or anoxia evoked by a decrease in the tension of inspired oxygen. In both situations, the diffusion properties of the nervous tissue are substantially changed. We can assume that the observed changes in the three diffusion parameters α, λ, and k' affect the diffusion of various substances—ions, neurotransmitters, metabolic substances, as well as various drugs used in therapy of nervous diseases—to the place of action or clearance. The narrow ECS ceases to disseminate neuractive substances or to allow exodus of metabolites. ECS volume shrinkage and increase in its tortuosity can aggravate the anoxia-related ionic changes, particularly K^+ accumulation and pH shifts, accumulation of excitatory amino acids, and accumulation of a variety of neurotransmitters such as glutamate and glycine (enhancing excitatory amino acid excitotoxicity and cell swelling), and other substances—factors that lead to ischemic brain damage (for review see Meldrum, 1985; Siesjö, 1988, 1990). It has also been shown that Ca^{2+} diffusion is influenced mainly by the tortuosity of the tissue, rather than other factors such as binding to extracellular charge sites or uptake (Nicholson and Rice, 1987). It is therefore reasonable to assume that the excessive ionic and ECS volume changes can contribute to irreversible tissue damage.

Knowledge about ECS diffusion parameters is also important for interpretation of in vivo microdialysis studies and of diffusion-weighted nuclear magnetic resonance imaging data—two methods currently used widely for studies of ischemic brain damage. Evaluation of results obtained during microdialysis must take into account differences between diffusion in calibration solution and in CNS tissue (Benveniste et al., 1989; Rice and Nicholson, 1991).

The TMA^+ diffusion studies deal with only the three diffusion parameters that regulate the migration of any substance in the nervous tissue. It must be remembered that the migration of substances might be influenced by other important factors, such as specific high-affinity uptake systems (Rice and Nicholson, 1991). The diffusion properties of TMA^+, as a relatively small ion with apparent molecular mass of 74 Da, can be compared particularly with those of biologically important ions and some neurotransmitters (e.g., acetylcholine, γ-aminobutyrate, glutamate). However, the diffusion parameters of substances with greater molecular mass, such as glucose (180 Da), ATP (500 Da), and various neurohormones and neuropeptides [e.g., dynorfin, substance P, galanin (1,000–3,000 Da), and nerve growth factor (~40,000 Da)], could be altered in both "early" and "later" stages of ischemia even more than the diffusion parameters of TMA^+.

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