Extracellular K^+, pH, and volume changes in spinal cord of adult rats and during postnatal development[1]

E. SYKOVÁ,[2] P. JENDELOVÁ, J. SVOBODA, AND A. CHVÁTAL

Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Czechoslovak Academy of Sciences, Bulovka, Pavilon 11, 180 85 Prague 8, Czechoslovakia

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Activity-related transient changes in extracellular K^+ concentration ($[K^+]_e$), extracellular pH (pH_e), and extracellular volume (EC volume) were studied by means of ion-selective microelectrodes in the adult rat spinal cord *in vivo* and in neonatal rat spinal cords isolated from pups 3–14 days of age. Repetitive electrical nerve stimulation (10–100 Hz) in adults elicited increases in $[K^+]_e$ by about 2.0–3.5 mM, followed by a poststimulation K^+ undershoot and triphasic alkaline–acid–alkaline changes in pH_e. In 3- to 6-day-old pups, the $[K^+]_e$ increased by as much as 6.5 mM at a stimulation frequency of 10 Hz, and this was accompanied by an alkaline shift. Increases in $[K^+]_e$ as large as 1.3–2.5 mM accompanied by an alkaline shift were evoked by a single electrical stimulus. Stimulation in 10- to 13-day-old pups produced smaller $[K^+]_e$ change and an acid shift, which was preceded by a small initial alkaline shift, as in adult rats. We conclude that glial cells buffer the activity-related $[K^+]_e$ increase and alkaline pH_e shifts. Mg^{2+} blocked the alkaline but not the acid shift. Acetazolamide had no effect on the alkaline shift but blocked the acid shift. The alkaline shift was enhanced and the acid shift blocked by Ba^{2+}, amiloride, 4-acetamido-4′-isothiocyanotostilbene-2,2′-disulfonic acid (SITS), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Activity-related acid shifts therefore have a complex mechanism, which includes Na^+/H^+ exchange, Cl^-/HCO_3^- exchange, or $Na^+/Cl^-/H^+/HCO_3^-$ antiport, $Na^+-HCO_3^-$ cotransport, and H^+ efflux through voltage-sensitive H^+ channels. Application of GABA evoked an alkaline shift in the pH_e baseline, which was blocked by picrotoxin. The activation of GABA-gated Cl^- channels, which induces a passive net efflux of bicarbonate, can therefore lead to an alkaline shift in pH_e. The poststimulation alkaline shifts were blocked by ouabain and reflect coupled clearance of K^+ and H^+ by active transport processes. The EC volume in the adult rat occupies about 20–25% of the tissue; in neonatal cord, 28–40%. Electrical or adequate stimulation-evoked ionic changes in adult rats were accompanied by shrinkage of the extracellular space by 20–50%. We conclude that the size of the EC volume is altered during stimulation of an afferent input, central nervous system development, and peripheral or central injury.

Key words: astrocytes, extracellular K^+, extracellular pH, extracellular volume, development, neuroglia, spinal cord, rat.

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On a examiné, dans la moelle épinière de rats adultes *in vivo* et dans les moelles épinière isolées de rats néonatals de 3–14 jours, les variations transitoires, reliées à l'activité, de la concentration de K^+ extracellulaire ($[K^+]_e$), du pH (pH_e) et du volume extracellulaire (volume EC), en utilisant des microélectrodes sensibles aux ions. Une stimulation nerveuse électrique répétitive (10–100 Hz) chez les adultes a provoqué des augmentations de $[K^+]_e$ d'environ 2,0–3,5 mM, suivies en post-stimulation d'une baisse de K^+ et de variations triphasiques alcalines–acides–alcalines du pH_e. Chez les ratons de 3–6 jours, la $[K^+]_e$ a augmenté de plus de 6,5 mM à une fréquence de stimulation de 10 Hz, et cette augmentation était accompagnée d'un décalage alcalin. Le stimulus électrique simple a induit des augmentations de $[K^+]_e$ de 1,3–2,5 mM, accompagnées de décalages alcalins. Une stimulation chez les ratons de 10–13 jours a provoqué une plus faible variation de $[K^+]_e$ et un décalage acide qui, comme chez les rats adultes, a été précédé d'un faible décalage alcalin initial. Nous concluons que les cellules gliales ont un effet tampon sur l'augmentation de $[K^+]_e$ et les décalages de pH_e alcalins associés à l'activité. Le Mg^{2+} a bloqué le décalage alcalin mais n'a pas bloqué le décalage acide. L'acétazolamide n'a pas eu d'effet sur le décalage alcalin mais a bloqué le décalage acide. Le décalage alcalin a été stimulé et le décalage acide bloqué par le Ba^{2+}, l'amiloride, l'acide disulfonique 4-acétamido-4′-isothiocyano-2,2′-stilbène (SITS) et l'acide disolfonique diisothiocyanostilbène (DIDS). Ainsi, les décalages acides reliés à l'activité relèvent d'un mécanisme complexe qui comprend l'échange Na^+/H^+, l'échange Cl^-/HCO_3^- ou l'antiport de $Na^+/Cl^-/H^+/HCO_3^-$, le cotransport de $Na^+-HCO_3^-$ et le transport de H^+ à travers les canaux H^+ sensibles à la tension. L'application de GABA a provoqué un décalage alcalin de la ligne de base du pH_e, décalage qui a été bloqué par la picrotoxine. L'activation des canaux Cl^- avec un mécanisme de porte activé par le GABA, qui induit un flux net passif de bicarbonate, pourrait ainsi mener à un décalage alcalin du pH_e. Les décalages alcalins post-stimulation ont été bloqués par l'ouabaïne et reflètent la clairance couplée de K^+ et de H^+ par les processus de transport actif. Le volume EC occupe environ 20–25% du tissu chez le rat adulte et 28–40% de la moelle épinière néonatale. Les variations ioniques produites par une stimulation appropriée ou une stimulation électrique chez les rats adultes ont été accompagnés d'une réduction de 20–50% de l'espace extracellulaire. Nous concluons que la dimension du volume EC est altérée durant la stimulation d'un influx nerveux afférent, le développement du système nerveux central et durant une lésion centrale ou périphérique.

Mots clés : astrocytes, K^+ extracellulaire, pH extracellulaire, volume extracellulaire, développement, névroglie, moelle épinière, rat.

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[2]Author to whom all correspondence should be addressed.

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Introduction

Any neuronal activity is accompanied by transmembrane ionic and water fluxes, resulting in prolonged transient extracellular ionic shifts and in changes in extracellular volume (EC volume). However, it was not until the last 10 years that extracellular space (ECS) came to be recognized as a dynamically changing microenvironment for the brain, with a significant influence on the function of neurones and glia (for reviews see Nicholson 1980; Syková 1983, 1991, 1992). There is increasing evidence that glial cells play a crucial role in extracellular ion homeostasis and volume (for reviews see Ballanyi and Grafe 1988; Walz 1989; Chesler 1990; Hertz 1990). It is at present accepted that the glial cells play an important role in extracellular K^+ homeostasis, as suggested originally by Hertz (1965) and Orkand *et al.* (1966). The role of glia in regulation of other extracellular ionic shifts and EC volume is less clear. However, recent findings also strongly support their role in the regulation of extracellular pH (pH_e) homeostasis.

The intimate relationship between neurones and glia is important for normal brain functioning and could be impaired in various disease states, including demyelinating diseases, anoxia, and central nervous system (CNS) injury. Astrocytes are the most abundant type of glial cells. They fill the space between the neurones, maintain the optimal distance between them, and form gap junctions with other astrocytes and close contacts with blood vessels. In addition to their role in ion and volume homeostasis, they apparently may have various types of other functions since they possess (at least in cultures) various kinds of ion channels and receptors for a multitude of neurotransmitters (Kimelberg 1988; Barres *et al.* 1990). They both receive and send humoral signals to and from local and systemic sites, e.g., cytokines and neuroptrophic and growth factors.

During development astrocytes undergo marked morphological changes from flat polygonal cells to process-bearing cells and typical changes in glial intermediate filaments reflected by intensity of glial fibrillary acidic protein (GFAP) staining. A typical reaction to injury and to various inherited or degenerative diseases of metabolic, immunologic, and infectious origin is reactive gliosis characterized by astrocyte proliferation and hypertrophy. We assume that immature or reactive astrocytes may not be able to ensure their homeostatic function (Ransom *et al.* 1985; Jendelová and Syková 1991). Consequently it is of great interest to find out how the ionic and volume homeostasis and the relationship between neurones and glia differ during CNS development and reactive gliosis.

This review deals with activity-related ionic and volume changes in spinal cord of adult rats and during development, with transient changes during peripheral stimulation and injury. It focuses on their mechanisms and on possible differences in roles of mature, immature, and proliferating glial cells *in vivo* in K^+, pH_e, and EC volume homeostasis.

Measurements of ionic and EC volume changes

The use of ion-sensitive microelectrodes has made it possible to record transient intracellular and extracellular changes in K and pH during neuronal activity *in vivo*. K^+ activity was recorded with double-barreled K^+-selective microelectrodes filled with a liquid ion exchanger (Corning 477317) prepared by a procedure described previously (Kříž *et al.* 1974). K^+-selective microelectrodes were calibrated in solutions containing 3, 4, 5, 6, 8, or 10 mM KCl in 150 mM NaCl. Basically the same procedure was adopted to prepare the double-barreled pH-sensitive microelectrodes (Chvátal *et al.* 1988; Syková and Svoboda 1990). In principle, the reference channel was filled with 0.15 M NaCl solution, while the pH-sensitive channel contained, in a siliconized tip, a 200- to 1000-μm column of liquid Hydrogen Ion Ionophore II-Cocktail A (Fluka). The backfilling solution was composed of (mM) KH_2PO_4, 40.0; NaOH, 23.0; and NaCl, 15.0 (pH 7.0). Electrode sensitivity was tested in standard solutions, the pH of which was 7.0, 7.2, 7.4, 7.6, 7.8, or 8.0 with a background of 150 mM NaCl and 3 mM KCl. The slope of the electrodes was about 57 mV/unit of pH change (at 22–25°C), and the electrodes had a resistance of 700–1800 Mohm. The electrical arrangements were the same as described for K^+-selective microelectrodes (Kříž *et al.* 1974). Each channel of a double-barreled microelectrode was connected to one input of a differential amplifier. Microelectrodes were inserted into the spinal cord using two micromanipulators.

Dynamic changes in the size of the ECS were studied by means of the iontophoretic administration of ions that do not cross the cell membranes and therefore remain in the ECS. Their concentration in the ECS is in inverse proportion to the size of the ECS. The ion exchange for K^+ (Corning 477317) is highly sensitive to tetraalkylammonium ions and to choline, which in small concentrations, are not toxic and do not cross cell membranes (Nicholson and Phillips 1981; Kříž and Syková 1981). Tetramethylammonium ions (TMA^+) were therefore used for testing changes in the size of the ECS. Using a current of 10 to 100 nA, these ions were administered for 5 to 60 s into the ECS with a single-channel or multichannel iontophoretic pipette. The tip of the K^+-selective microelectrode (K^+-ISM) with which we recorded TMA^+ concentration changes during administration was 80 to 300 μm distant from the tip of the iontophoretic pipette. To determine changes in the actual volume fraction (α) and tortuosity (λ), the distance needs to be known exactly. The two electrodes were therefore glued together with cement, and the intertip distance was measured exactly with a microscope. After they had been joined together, the electrodes were introduced into the nervous tissue. The ions were administered at regular intervals (2–10 min), always with the same current. At least 3 identical control curves were obtained in succession before the stimulation was applied. Only those experiments in which three subsequent control curves did not differ by more than 5% were included for analysis. The relative changes in the size of the ECS were computed from the changes in the concentration of the given ions, according to the formula

$$[1]\quad \underset{(\%\ \text{control})}{\text{Decrease in ECS}} = \left(1 - \frac{\Delta[TMA^+]\ \text{before activity}}{\Delta[TMA^+]\ \text{after activity}}\right) \times 100$$

For computing and determining absolute values of α and λ we iotophoresed the ions into the nervous tissue for 50 to 60 s. Before and after measurements in the nervous tissue the diffusion curve was recorded in 0.3% agar. Measurement of the concentration change in the agar gives the free-diffusion values for the relevant ion, in which $\alpha = 1$ and $\lambda = 1$. K^+-ISMs were calibrated in appropriate calibration solutions containing TMA^+ and K^+ (see Svoboda and Syková 1991). The α and λ in the spinal cord were determined by computation after Nicholson and Phillips (1981). The results of these studies show that if we incorporate factors α and λ in Fick's

law, diffusion in the CNS is described fairly satisfactorily. The micropipette from which TMA^+ was released iontophoretically acts as the source $Q = In/F$. The appropriate solution of the diffusion equation is

$$[2] \quad C = ((In\lambda^2)/(4\pi FDr\alpha)) \text{ erfc } (r\lambda/2\ (Dt)^{1/2})$$

where C is the concentration, In is the iontophoretic current increment, n is the electrode transport number, F is the Faraday's electrochemical equivalent, r is the distance of ISM from the ion source (iontophoretic pipette), D is the diffusion coefficient, t is time of the probe ion application, and erfc is the complementary error function.

In vivo experiments were performed on adult Wistar rats, which were anesthetized with pentobarbital (40–60 mg/kg). A laminectomy was performed between L_2 and L_6. The animals were artificially ventilated and paralyzed (for details see Svoboda *et al.* 1988; Syková and Svoboda 1990). *In vitro* experiments were performed on 3- to 14-day-old rat pups. Animals were decapitated, and the lumbosacral spinal cord was dissected in a chamber with cold (9–11°C) modified Ringer's solution of the following composition: NaCl, 113.0 mM; KCl, 3.5 mM; $CaCl_2$, 2.0 mM; Na_2HPO_4, 2.0 mM; $NaHCO_3$, 28 mM; and glucose, 1 g/L. The isolated cord was placed in a small chamber, and the preparation was continuously perfused with Ringer's solution. During 1–2 h the temperature was increased to 21–23°C. The solution was saturated with 95% O_2 and 5% CO_2 (pH 7.3–7.35).

Microelectrodes were inserted into the spinal cord from its dorsal surface. The dorsal root of the same segment or a peripheral nerve was stimulated supramaximally (rectangular pulses of 5 V or less; duration 0.1 ms) with fine bipolar silver electrodes. Transcutaneous electrical nerve stimulation was delivered through a pair of stainless-steel needles inserted through the skin below the plantar surface. Natural stimuli (touch, press, heat) were applied to the most sensitive area of the receptive field (for details see Syková and Svoboda 1990).

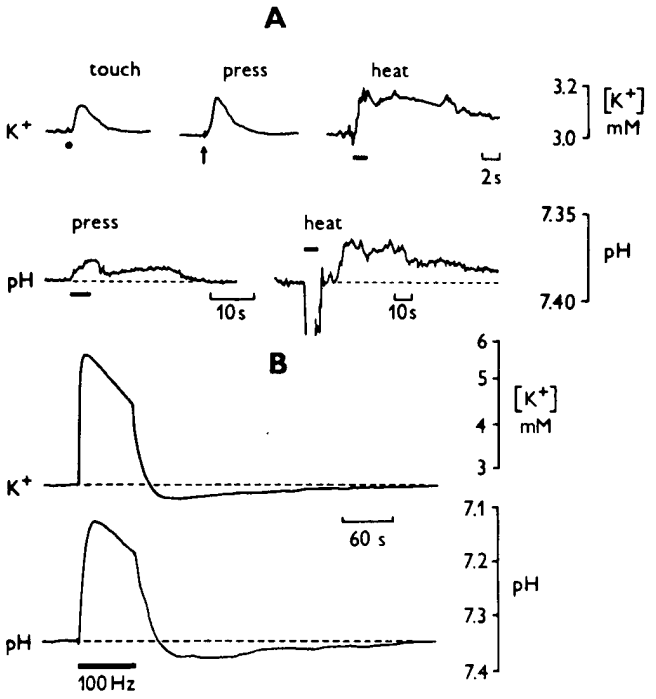

FIG. 1. (A) Transient increase in $[K^+]_e$ and decrease in pH_e in the L_4 spinal segment in response to adequate stimuli applied to the plantar region of the ipsilateral hind paw. The K^+- and pH-sensitive microelectrodes were inserted to a depth of 600 μm from the dorsal surface. The curves represent responses to light touch, strong press, and application of about 1 mL of hot water onto the hind limb (heat). Horizontal bars indicate stimulus duration. $[K^+]_e$ and pH_e data are different experiments. (B) $[K^+]_e$ and pH_e changes in dorsal horn of segment L_4 of rat spinal cord evoked by repetitive bipolar electrical stimulation with acupuncture needles (100 Hz, 60 s) in plantar muscles of the ipsilateral hind paw. Horizontal bar indicates stimulus duration. Two K^+- and pH-sensitive microelectrodes were inserted separately from dorsal spinal surface. Recordings from a depth of 600–700 μm. (Adapted from Svoboda *et al.* (1988) and Syková and Svoboda (1990).)

K^+ and pH_e changes in adult rat

Almost all kinds of stimulation, electrical or natural, lead to transient increases in $[K^+]_e$ and alkaline–acid shifts in pH_e in the corresponding spinal cord segments. The main source of changes in $[K^+]_e$ are stimulated neurons, unmyelinated fibres, and unmyelinated terminals of axons (for reviews see Syková 1983, 1992). An increase of 0.1–0.2 mM has been found in spinal cord after a single electrical stimulus applied to peripheral nerves or after a single adequate stimulus (e.g., light touch or pinch) applied to the skin (Fig. 1A). A sustained increase in $[K^+]_e$ in the spinal dorsal horn of the rat was evoked by chemical and thermal injury of the hind paw (Svoboda *et al.* 1988). Repetitive electrical stimulation or prolonged adequate stimulation leads to summation of responses to a certain level, the so-called ceiling level, from a baseline ("resting") level of about 3–4 mM to as much as 6–12 mM (Fig. 1B). No further changes in $[K^+]_e$ are found because a steady state is established, which is a result of concurrent release and clearance of K^+ (Fig. 1B). This ceiling level (Kříž *et al.* 1975; Heinemann and Lux 1975) in the CNS is only broken through by pathological events, e.g., epileptic activity, anoxia, spreading depression, and application of convulsive drugs, and is higher in immature nervous tissue, i.e., in situations when K^+ homeostasis is impaired.

K^+ homeostasis is ensured by two main mechanisms: (*i*) activation of the Na/K pump in neurones and glia and (*ii*) glial cell K^+ uptake or buffering of extracellular rise in $[K^+]_e$ (Syková 1983, 1992; Walz 1989). Active neurones as well as primary fibres lose K^+ and induce a change in the ionic composition of the ECS (Syková and Vyklický 1977). This may be an important way in which neurones interact and integrate their activity; however, stability of nervous tissue function requires fast renewal of the ECS ionic composition. The conclusion that K^+ is cleared by means of active transport is strongly supported, since there is a poststimulation decrease below resting level, the so-called K^+ undershoot (Fig. 1B), which can be blocked by inhibitors of the Na/K pump (e.g., ouabain), or by anoxia, ischemia, or anaesthetic drugs (Kříž *et al.* 1975; Syková *et al.* 1985). Glial cells also significantly contribute to activity-related clearance of K^+ increase in ECS. There is evidence that K^+ clearance by glia include uptake of K^+ driven by the Na/K pump (Hertz 1965) and K^+ spatial buffering (Orkand *et al.* 1966; Syková *et al.* 1988).

Concomitantly with an increase in $[K^+]_e$ any neuronal activity results in a transient change in ECS acid–base balance. In adult animals the activity-related pH_e changes have a typical time course: alkaline–acid shift during stimulation, which

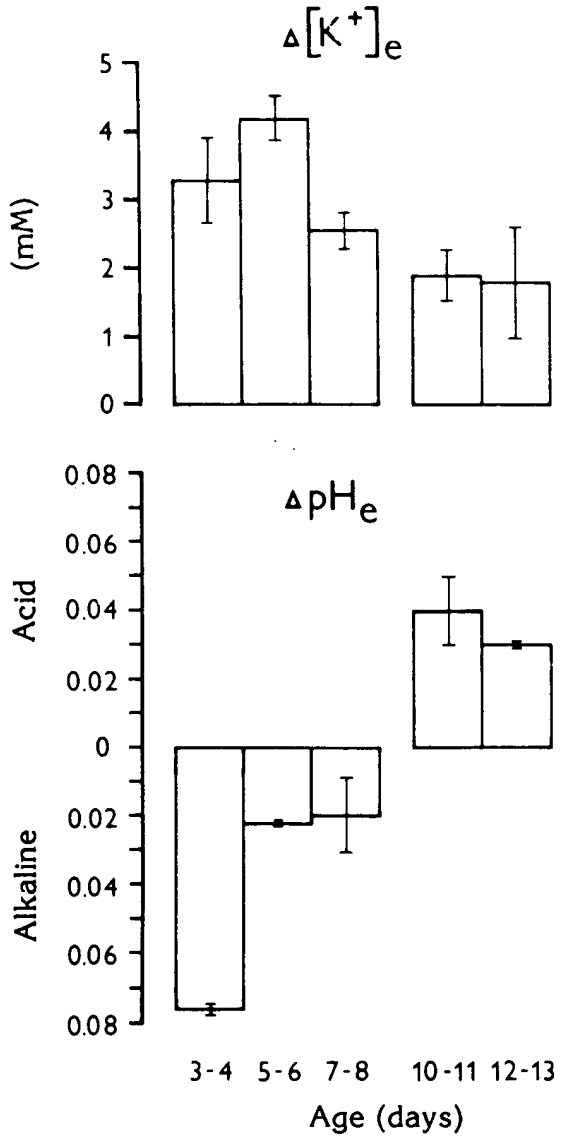

FIG. 2. $[K^+]_e$ and pH_e changes in the spinal dorsal horn in rats 3 to 13 days old. Each column represents data from animals 3 to 4 days old (n = 6); 5 to 6 days old (n = 4); 7 to 8 days old (n = 4); 10 to 11 days old (n = 4); and 12 to 13 days old (n = 3) (mean ± SEM). All recordings at a depth of about 200 μm; stimulation frequency 10 Hz. (Jendelová and Syková 1991, reprinted with permission.)

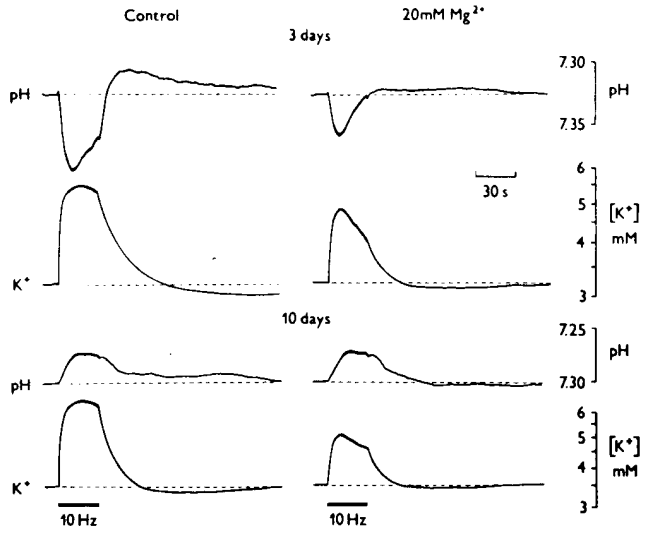

FIG. 3. (Left) Stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of rats 3 and 10 days old. Note that the stimulation of the dorsal root at a frequency of 10 Hz evoked an alkaline shift in the 3-day-old pup, which was accompanied by an increase in $[K^+]_e$; when stimulation was discontinued, the poststimulation acid shift of smaller amplitude appeared, which was accompanied by a K^+ undershoot. In the 10-day-old rat there was a slight initial alkaline shift, which was followed by an extracellular acid shift. (Right) Effect of 20 mM Mg^{2+} on pH_e and $[K^+]_e$ in the spinal dorsal horn of rats 3 and 10 days old. Note decrease of the alkaline shift, decrease of the poststimulation acid shifts, and decrease of $[K^+]_e$ changes. Only the acid shift that occurred during stimulation in the 10-day-old rat remained unaffected. (Jendelová and Syková 1991, reprinted with permission.)

is followed in mammalian spinal cord by poststimulation alkaline undershoot (Fig. 1B; Syková 1989; Syková and Svoboda 1990) and in the frog spinal cord by delayed acid shift (Chvátal *et al.* 1988). The pH_e resting level recorded in various areas of adult CNS varies between 7.1 and 7.3, i.e., pH_e is slightly alkaline but more acid than that in blood or cerebrospinal fluid (CSF). In spinal cord of the rat regional differences in pH_e were found in unstimulated rat spinal cord. The pH_e in the lower dorsal horn was about 7.15, i.e., lower by about 0.2 pH units than that measured in the CSF. In the upper dorsal horn and in the ventral horn the pH_e baseline was only slightly more acid (7.25−7.35) than in supraspinal fluid (7.35−7.40) (Syková and Svoboda 1990). An alkaline shift in pH_e baseline of 0.05−0.10 pH units was found after blockade of synaptic activity by applying Mn^{2+} in a concentration of 4−5 mM onto the spinal cord surface. The variations in pH_e baseline had a similar depth profile as the variations in $[K^+]_e$ baseline in unstimulated spinal cords. It has been demonstrated previously that increases in $[K^+]_e$ baseline found in the lower dorsal horn are associated with a high level of spontaneous activity of dorsal horn neurones as a result of continuous excitatory input from the periphery (Svoboda *et al.* 1988).

Transient acid shifts in pH_e by 0.01−0.05 pH units were found when acute nociceptive stimuli (pinch, press, heat) were applied to the hind paw (Fig. 1A). Chemical or thermal injury evoked by subcutaneous injection of turpentine or by application of 1−3 mL of hot oil onto the hind paw produced a long-term decrease in pH_e baseline in the lower dorsal horn by about 0.05−0.10 pH units (Syková and Svoboda 1990). The decrease in pH_e began 2−10 min after injury and persisted for more than 2 h. Electrical nerve stimulation (10−100 Hz, 20−60 s) elicited triphasic (alkaline−acid−alkaline) changes in pH_e, which had a similar depth profile as the concomitantly recorded increase in $[K^+]_e$ (Fig. 1B).

An initial alkaline shift by about 0.01 pH units was found to be decreased by La^{3+}, an H^+ channel blocker (Syková and Svoboda 1990). It has been shown that acid extrusion in CNS neurons and glia involves amiloride-sensitive Na^+/H^+ exchange, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) sensitive and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) sensitive Cl^-/HCO_3^- exchange, $Na^+/Cl^-/H^+/HCO_3^-$ antiport, and $Na^+-HCO_3^-$ cotransport (for reviews see Chesler 1990; Syková 1992). Stimulation-evoked acid shifts in spinal cord were blocked by amiloride, SITS, DIDS, and La^{3+} and therefore have a complex mechanism, which may include Na^+/H^+ exchange, Cl^-/HCO_3^-

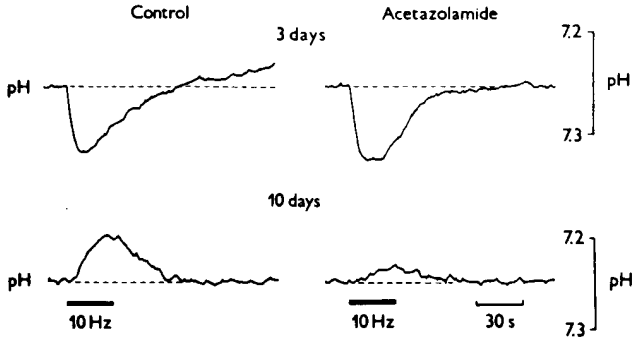

FIG. 4. Effect of acetazolamide (5 mM) on pH_e changes in the spinal dorsal horn of rats 3 and 10 days old. pH_e changes were evoked by stimulation at a frequency of 10 Hz. Note that the alkaline shift was not changed, whereas the acid shifts during and after stimulation were substantially decreased. (Jendelová and Syková 1991, reprinted with permission.)

exchange or $Na^+/Cl^-/H^+/HCO_3^-$ antiport, $Na^+-HCO_3^-$ cotransport, and H^+ efflux through voltage-sensitive H^+ channels. Superfusion of isolated frog spinal cord with NaF, which blocks the glycolitic portion of metabolic processes and production of lactate, had no effect on stimulation-evoked alkaline–acid changes in ECS (Syková *et al.* 1988). The poststimulation alkaline shift ("alkaline undershoot") was blocked by ouabain, disappeared during anoxia, and reflects, therefore, coupled clearance of K^+ and H^+ by active transport processes (Syková and Svoboda 1990).

K^+ and pH_e changes during postnatal development

The transient changes in K^+ and pH_e homeostasis, their mechanisms, and the role of glia in K^+ in pH homeostasis have been investigated during postnatal development. Stimulation-evoked transient changes in $[K^+]_e$ and pH_e were studied in the neonatal rat spinal cord isolated from 3- to 14-day-old pups (Jendelová and Syková 1991). In unstimulated pups the $[K^+]_e$ baseline was elevated and pH_e was more acid than that in Ringer's solution (3.5 mM K^+, pH 7.3–7.35). In 3- to 6-day-old pups the actual $[K^+]_e$ value in spinal dorsal horn was 3.91 ± 0.12 mM and pH_e was 7.19 ± 0.01 (n = 14, SEM). The actual values of $[K^+]_e$ in 10- to 14-day-old pups were significantly higher than those in 3- to 6-day-old animals, i.e., 4.35 ± 0.15 mM (n = 10, $P < 0.05$). The pH_e in 10- to 14-day-old pups was 7.11 ± 0.01 (n = 10), i.e., it was significantly more acid than in 3- to 6-day-old animals ($P < 0.01$).

In neonatal rat spinal cord the stimulation-evoked changes in $[K^+]_e$ were much larger than in adult animals. The maximal changes of 2.1–6.5 mM were found at a stimulation frequency of 10 Hz in 3- to 6-day-old animals. In older animals the $[K^+]_e$ changes progressively decreased (Fig. 2; Jendelová and Syková 1991). We found that in 3- to 13-day-old pups the $[K^+]_e$ changes evoked by a single electrical stimulus are as large as those evoked in the adult rat spinal cord with stimulation at a frequency 10–30 Hz (Svoboda *et al.* 1988), i.e., by as much as 1.5–2.5 mM.

In 3- to 8-day-old pups, the stimulation evoked an alkaline shift by 0.1–0.2 pH units, which was followed by a smaller poststimulation acid shift by 0.03–0.07 pH units (Fig. 3). In 10- to 14-day-old rats, however, the stimulation evoked an acid shift which was preceded by a scarcely discernible alkaline shift as in adult animals (Syková and Svoboda 1990). $MgCl_2$ at 20 mM reversibly reduced the alkaline but not the acid shift by 50–60% (Fig. 3). Bath application of the carbonic anhydrase inhibitor, acetazolamide, either had no effect or slightly enhanced the alkaline shift, whereas the acid shift decreased by 70–80% (Fig. 4).

The alkaline shift was enhanced in Hepes-buffered saline; it was significantly decreased by La^{3+}, a H^+ channel blocker, and by picrotoxin in concentrations from 10^{-7} M (Syková *et al.* 1992). At $10^{-7}-10^{-6}$ M, picrotoxin had no effect on K^+ and pH_e resting level. Superfusion of spinal cord with GABA (10^{-3} M) elicited an extracellular alkaline shift (Syková *et al.* 1992), which was blocked by picrotoxin. We also found that GABA at $10^{-3}-10^{-2}$ M increases $[K^+]_e$. However, the alkaline shift evoked by superfusion of GABA in 3- to 14-days-old pups cannot be explained by an increase in $[K^+]_e$. We found in experiments with superfusion of the spinal cord with Ringer's solution containing elevated K^+ that an imposed increase in $[K^+]_e$ is accompanied by an acid shift in pH_e baseline (Syková 1978; Jendelová and Syková 1991). In nominally HCO_3^--free solution, buffered with Hepes, GABA evoked no alkaline shift. Our results suggest that the activation of GABA-gated Cl^- channels in spinal cord can give rise to an HCO_3^- efflux, which can explain the pH_e alkaline shifts observed in the dorsal horn during a stimulation of an afferent input (see also Kaila and Voipio 1987; Chen and Chesler 1991).

In the spinal cord of immature rat Ba^{2+}, which blocks K^+ channel permeability, enhanced the stimulation-evoked alkaline shift and K^+ ceiling level (Syková *et al.* 1992). Similarly, Ba^{2+} enhanced the initial alkaline shift in rat cortex (Chesler and Kraig 1989). The observed effect of Ba^{2+} on the alkaline shift may be generated by acid influx into neurones through a channel-mediated pathway (Endres *et al.* 1986; Chesler and Chan 1988). The relationship of the glial stimulation-evoked intracellular akaline shifts to the extracellular alkaline shift is unclear. Ba^{2+} induces hyperpolarization of glia in *Necturus* optic nerve and increases $Na^+-HCO_3^-$ cotransport into glial cells (Astion *et al.* 1987, 1989). Increase in $Na^+-HCO_3^-$ cotransport cannot give rise to interstitial alkalinization, but it can contribute to the observed stimulation-evoked acid shifts.

As in adult rats the acid shift in immature rats was blocked by amiloride and SITS and therefore has a complex mechanism which may include Na^+/H^+ exchange, $Na^+/Cl^-/HCO_3^-/H^+$ exchange, and $Na^+-HCO_3^-$ cotransport, the membrane transport systems that have been demonstrated not only in neurones but also in glia (for reviews see Chesler 1990; Syková 1992). It has been demonstrated recently that mammalian astrocytes actively regulate their pH_i by acid extrusion membrane transport processes which are dependent on Na^+, Cl^-, and HCO_3^-. Since gliogenesis occurs during the first 10 days postnatally, the lumbosacral region of 1-day-old pups was X-irradiated (4000 R) to block gliogenesis (Gilmore 1963). In irradiated pups the enhanced $[K^+]_e$ changes and the alkaline shifts persisted even in 10- to 14-day-old pups (Syková *et al.* 1992). Since carbonic anhydrase and gliogenesis to a great extent occur postnatally with their peak at about 10 days, it can be concluded that in spinal cord (Jendelová and Syková 1991) as in the optic nerve (Ransom *et al.* 1985), the stimulation-evoked acid shifts are related to gliogenesis. These results suggest that glial cells buffer the activity-related $[K^+]_e$ increase and alkaline pH_e shifts.

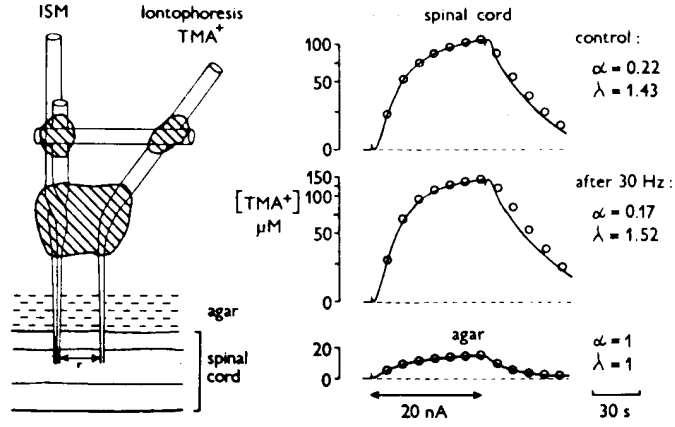

FIG. 5. Method of determining EC volume fraction (α) and tortuosity (λ) and TMA^+ diffusion curves in spinal cord *in vivo*. (Left) Schematic drawing of the experimental arrangement for diffusion measurements. Two microelectrodes, the double-barreled microelectrode sensitive to K^+ and to TMA^+ (ISM) and the micropipette for TMA^+ iontophoresis, were glued together with dental cement at the area of their shanks to stabilize the intertip distance (r) and with glass tube bridge connecting the two microelectrodes at their upper end. (Right) The diffusion curves were recorded prior to (control) and 9 min after repetitive electrical stimulation at a frequency of 30 Hz for 2 min. Diffusion curve after stimulation is larger, as a result of the EC volume decrease. Before stimulation (control) $\alpha = 0.22$ and $\lambda = 1.43$; after stimulation $\alpha = 0.17$ and $\lambda = 1.52$. Diffusion parameters were $I = 20$ nA; $r = 180$ μm; $n = 0.308$; $D = 1.33 \times 10^{-9}$ m^2 s^{-1}. (Adapted from Svoboda and Syková 1991.)

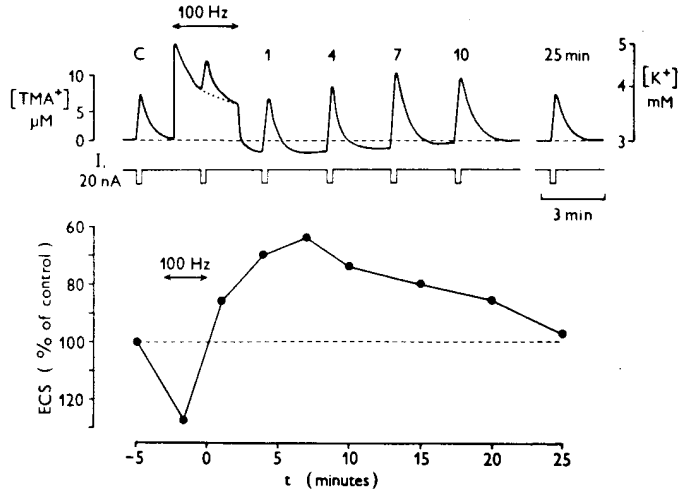

FIG. 6. Effect of repetitive electrical stimulation (3 min, 100 Hz) on the EC volume in spinal dorsal horn of adult rat. (Upper trace) The record from the chart recorder comprises the diffusion curves at a depth of 500 μm evoked by the regular iontophoretic application of TMA^+ (application at 3-min intervals) prior to stimulation, during the stimulation-evoked increase in $[K^+]_e$, and during the poststimulation K^+ undershoot below the original $[K^+]_e$ baseline. TMA^+ applications for 15 s, iontophoretic current (I) = 20 nA, and the intertip distance $r = 240$ μm. Dotted line represents the predicted time course of the $[K^+]_e$ curve without iontophoretic application and broken line the prestimulation $[K^+]_e$ baseline. (Lower trace) Graph of the EC volume changes expressed as percentage of control. (Adapted from Svoboda and Syková 1991.)

Changes in EC volume and tortuosity

It has been recognized that during depolarization and during neuronal activity the ECS can reversibly decrease as a result of the activity-related cell swelling. Experimental elevation of $[K^+]_e$ by KCl application leads to membrane depolarization of various brain cells, to an increase in the volume of the cells, and to a compensatory EC volume decrease as a result of cellular accumulation of ions (particularly K^+, Na^+, Cl^-, and HCO_3^-) and of water. K^+-induced swelling is mostly attributed to glia, particularly to astrocytes (Kimelberg and Ransom 1986), but swelling of neuronal elements has also been shown (Ritchie *et al.* 1981; Trubatch *et al.* 1977; Tasaki and Iwasa 1982). These previous studies, however, did not furnish any information on the size of the EC volume changes or on their dynamics. It should be noted that the "compensatory EC volume decrease," known also as regulatory volume decrease (RVD), has been seen only in cultured cells to date. It is not yet known if it happens in the intact CNS during hypoosmotic stress.

A method for measuring the dynamic changes in the EC volume fraction (α) and tortuosity (λ, a measure of the total path length that a diffusing particle travels because of the presence of obstructions in ECS) at a specific point in a nervous tissue has been developed recently (Nicholson and Phillips 1981; Nicholson and Rice 1988). With this method either relative changes in the EC volume or the absolute value of the ECS volume and tortuosity were found in cortex, spinal cord, optic nerve, and receptor organs during activity evoked by electric as well as adequate stimulation (Dietzel *et al.* 1980, 1982; Orkand *et al.* 1984; Ransom *et al.* 1985; Syková 1987; Svoboda and Syková 1991) as well as during epileptic activity (Heinemann and Dietzel 1984; Lehmenkühler *et al.* 1991) and during ischemia or spreading depression (Nicholson and Rice 1988).

The absolute values of EC volume and its tortuosity are of great interest, particularly during physiologically evoked neuronal activity or during various disease states in which the volume regulation in CNS by astrocytes can be impaired, or in which the astrocytes might not be able to ensure EC volume stability when there is an inflammation and damage in blood brain barrier (BBB). It is evident that changes in the EC volume may be either the cause or the result of the sustained hypo- or hyper-excitability due to synaptic hypo- or hyperactivity in CNS (Syková 1992).

The EC volume fraction, α, and tortuosity, λ, were examined in the spinal dorsal horns of adult rats and during postnatal development. The α and λ in spinal cord vary in unstimulated adult animals, with α between 20 and 25% of total spinal cord volume and λ about 1.5 (Svoboda and Syková 1991). These values are not significantly different from those found in brain by Nicholson and colleagues (Nicholson and Phillips 1981; Nicholson and Rice 1988). However, using the same mehtod, McBain *et al.* (1990) found that EC volume is only 12% in the CA1 pyramidal cell layer of hippocampus. Therefore neuronal packing is probably an important factor that determines the EC volume. In spinal cord EC volume was not significantly different throughout the dorsal and ventral horn gray matter.

Repetitive electrical stimulation of peripheral nerves at 3–100 Hz increased ECS volume in Rexed laminae III–V by 15.8 ± 2.7%, i.e., during the electrical stimulation EC volume increased apparently as a result of shrinkage of some neural elements (Svoboda and Syková 1991). However, after the end of stimulation, when the $[K^+]_e$ decreased below the

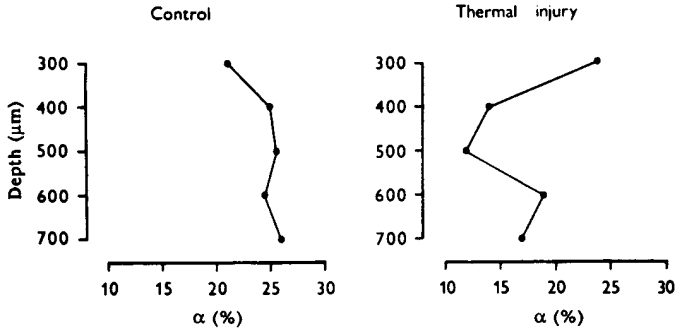

FIG. 7. Effect of thermal injury of the hind paw on the EC volume at various depth of the spinal dorsal horn. (Left) Typical EC volume fraction (α) at various depths of the spinal dorsal horn in the non-stimulated animal before injury. (Right) The EC volume decreased at depth of 400–700 μm. The maximal EC volume decrease from about 24 to about 12% was found at a depth of 500 μm 10–25 min after the thermal injury. (Svoboda and Syková 1991, reprinted with permission.)

original baseline (during K^+ undershoot), the EC volume decreased by 20–40%, i.e., to about 14–17% of total spinal cord volume (Fig. 5). The EC volume decrease was maximal at 2–10 min after the stimulation had been discontinued, and it returned to the prestimulation value in 15–40 min (Fig. 6). The decrease in the EC volume had the same depth profile as the ionic (K^+, Ca^{2+}, and pH_e) changes evoked by neuronal activity. The higher the stimulation frequency and intensity and the greater the concomitant ionic changes, the greater was the decrease in EC volume. The EC volume decrease persisted after stimulation had been discontinued. It regularly lasted longer than the stimulation-evoked increase in $[K^+]_e$, its time course being related to the poststimulation K^+ undershoot (Fig. 6) and poststimulation acid shift (Syková and Svoboda 1990).

The EC volume decreased by 20–50% (but no more than to 12% of total brain volume) after injury of the ipsilateral hind paw evoked either by subcutaneous injection of turpentine or by thermal injury (Svoboda and Syková 1991). The maximal changes were found in dorsal horn Rexed laminae III–V, 5–10 min after injection of turpentine and 10–25 min after thermal injury (Fig. 7), and persisted for more than 120 and 60 min, respectively. The tortuosity of the ECS was not significantly altered by stimulation or injury. These measurements indicate that the normal volume of the ECS in adult rats occupies one-fifth to one-quarter of total CNS volume, that the EC volume is larger in immature rats (about one-third of total CNS volume), and that significant dynamic changes in the EC volume accompany transmembrane ionic shifts resulting from neural activity.

We studied EC volume changes during development in rats 3- to 14-days-old, i.e., during the period of extensive gliogenesis (Svoboda *et al.* 1992*a*; E. Syková and A. Chvátal, unpublished observations). As observed in unmyelinated rat optic nerve (Ransom *et al.* 1985), we found that the ECS in spinal cord and in cortex of immature rats is larger, varying between 28 and 40% and decreasing with the animal age.

The mechanisms of cellular shrinkage and swelling during neuronal activity are discussed in several papers (for reviews see Kempski 1986; Kimelberg and Ransom 1986; Syková 1992). The mechanisms include (*i*) extracellular ionic changes (H^+ and K^+) accompanied by uptake of Na^+, Cl^-, and water; (*ii*) increase in intraneuronal osmolarity and (or) local extracellular decrease in osmolarity; (*iii*) glial spatial buffer mechanism (glial swelling due to increase in intracellular particles accompanied by water; (*iv*) glial swelling due to metabolic alterations such as acid–base balance; and (*v*) increase in intraneuronal particles, produced by enhanced metabolism during excessive neuronal activity. It is evident that the glial cells, and particularly astrocytic swelling, could play the important role in activity-related ECS volume changes. However, there is no direct evidence for astrocytic swelling in mammalian CNS *in vivo*.

Physiological significance of ionic and EC volume changes

The physiological significance of K^+, pH_e, and EC volume changes has been of great interest. It is evident that transient ionic changes in the nerve cell microenvironment might be a powerful mechanism in the regulation and integration of CNS function. Changes in $[K^+]_e$ (and also in $[Ca^{2+}]_e$) might serve as a signal. K^+ and Ca^{2+} can influence neuronal excitability, transmitter release, intercellular interaction, and glial cell function (for reviews see Somjen 1979; Nicholson 1980; Syková 1983). It is assumed that the increase in $[K^+]_e$ that is associated with repetitive neuronal activity reduces transmitter release from presynaptic terminals by curtailing the presynaptic spike amplitude by presynaptic depolarization (Kříž *et al.* 1975; Czéh *et al.* 1981). Moreover this effect of increased $[K^+]_e$ could be enhanced by a concomitant decrease in $[Ca^{2+}]_e$. In addition to its presynaptic action, the increase in $[K^+]_e$ influences impulse transmission postsynaptically, by depolarization of neurones and glia. K^+-mediated depolarization of postsynaptic membranes enhances neuronal excitability, which under some circumstances can dominate over its presynaptic inhibitory effect. K^+ accumulation in ECS therefore could act as a negative–positive feedback control system. The $[K^+]_e$ changes, determined by neuronal release, clearance, and by glial cell (particularly astrocyte) redistribution, constitute another way by which neurones and astrocytes can communicate. Astrocytes exhibit a potassium-induced, non-calcium-dependent release of glutamate, which can exert an action on neuronal glutamate receptors, and they exhibit higher activity of the enzymes glutamine synthetase and pyruvate carboxylase (Norenberg *et al.* 1988). An existence of glutamate–glutamine cycle between neurones and astrocytes may serve neuronal–astrocytic interaction (Hertz 1990). The role of K^+ accumulation and altered K^+ homeostasis during CNS development, in the modulation of neuronal activity and glial cell function, in states of CNS hypo- and hyper-excitability, in learning and memory formation, and during pathological states is far from clear, and it is closely related to the role of glia.

Activity-related dynamic changes in pH_e can be another important modulating and controlling factor in the CNS. Changes in pH_e of only a few tenths of a pH unit have been shown to evoke marked effects on neuronal ionic channels (Iljima *et al.* 1986; Konnerth *et al.* 1987), to affect neuronal excitability (Gruol *et al.* 1980; Balestrino and Somjen 1988), cell to cell coupling (Roos and Boron 1981) and glial cell membrane properties (for reviews see Walz 1989; Chesler 1990).

Significant changes in EC volume which occur in the CNS during physiological conditions, development, and in pathological states (Svoboda *et al.* 1992*a*, 1992*b*) may in addition

to ionic changes in the ECS influence neuronal excitability by affecting concentration of transmitters in synaptic clefts, migration of transmitters, peptides and ions in ECS, properties of ionic channels, and glial cell function. Indeed, changes in the shape and swelling of dendritic spines have been found to occur in high K^+ or after extensive stimulation and can be the basis of long-term potentiation (Van Harreveld and Fifkova 1975). An increase in synaptic efficiency due to the ECS shrinkage (produced either by swelling of astrocytes and (or) neuronal elements) may also be one of the mechanisms leading to sustained excitability increase, plastic neuronal changes, and influence protheosynthesis and memory formation.

Conclusions

Elevations in $[K^+]_e$ are associated with neural activity evoked by various kinds of stimulation. The main sources of K^+ are stimulated neurones, unmyelinated terminals, and fibres. The reason for the increase is obvious, as K^+ is released with each impulse, and therefore ongoing activity results in substantial K^+ accumulation in ECS.

Recent studies have revealed that the pH_e level is related to neuronal depolarization, metabolic activity, ion shifts across neuronal membranes, and glial cell buffering of pH_e changes. The time course and mechanisms of activity-related alkaline–acid shifts have been studied under physiological and pathological conditions. Current research suggests that activity-related alkaline shifts are due to (*i*) channel-mediated fluxes of bicarbonate and (*ii*) channel-mediated acid influx into neurones; while the acid shifts are due to (*i*) classic acid extrusion membrane transport systems (Na^+/H^+ or $Na^+/Cl^-/H^+/HCO_3^-$), (*ii*) $Na^+-HCO_3^-$ cotransport into glial cells, and (*iii*) efflux of lactic acid from metabolically active cells. Both the neurones and glial cells (oligodendrocytes as well as astrocytes) have been shown to possess several membrane transport systems which transport ions, including H^+ and HCO_3^-. It is reasonable to assume that glial cells regulate pH_e homeostasis at the expense of their intracellular pH. We therefore suggest that activity-related alkaline shifts in pH_e, as well as the excessive increase in $[K^+]_e$, are effectively buffered by glial cells.

Concomitantly with changes in ionic composition the size of the ECS is significantly altered during stimulation-evoked neuronal activity, during CNS development, and during peripheral or central injury. Immature astrocytes (and possibly astrocytes during proliferation and hypertrophy) are not able to ensure K^+, pH_e, and EC volume homeostasis. We conclude that changes in ECS ionic composition and volume may significantly influence the function of neurones, glia, and CNS excitability.

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