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K^+ and pH homeostasis in the developing rat spinal cord is impaired by early postnatal X-irradiation

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Activity-related transient changes in extracellular K^+ concentration ($[K^+]_e$) and pH (pH_e) were studied by means of ion-selective microelectrodes in neonatal rat spinal cords isolated from pups 2–14 days of age. Pups 1 to 2 days old were X-irradiated to impair gliogenesis and spinal cords were isolated 2–13 days postirradiation (PI). In 2- to 14-day-old pups PI stimulation produced ionic changes that were the same as those in 3- to 6-day-old control (non-irradiated) pups; e.g. the $[K^+]_e$ increased by 4.03 ± 0.24 mM (mean ± S.E.M., $n = 30$) at a stimulation frequency of 10 Hz and this was accompanied by an alkaline shift of 0.048 ± 0.004 pH units (mean ± S.E.M., $n = 32$) pH units. By contrast, stimulation in non-irradiated 10- to 14-day-old pups produced smaller $[K^+]_e$ changes, of 1.95 ± 0.12 mM (mean ± S.E.M., $n = 30$), and an acid shift of 0.035 ± 0.003 pH units which was usually preceded by a scarcely discernible initial alkaline shift, as is also the case in adult rats. Our results show that the decrease in $[K^+]_e$ ceiling level and the development of the acid shift in pH_e are blocked by X-irradiation. Concomitantly, typical continuous development of GFAP-positive reaction was disrupted and densely stained astrocytes in gray matter of 10- to 14-day-old pups PI revealed astrogliosis. In control 3- to 6-day-old pups and in pups PI the stimulation-evoked alkaline, but not the acid, shift was blocked by Mg^{2+} and picrotoxin (10^{-6} M). The acid shift was blocked, and the alkaline shift enhanced, by acetazolamide, Ba^{2+}, amiloride and SITS. Application of GABA evoked an alkaline shift in the pH_e baseline which was blocked by picrotoxin and in HEPES-buffered solution. By contrast, the stimulus-evoked alkaline shifts were enhanced in HEPES-buffered solutions. The results suggest a dual mechanism of the stimulus-evoked alkaline shifts. Firstly, the activation of GABA-gated anion (Cl^-) channels induces a passive net efflux of bicarbonate, which may lead to a fall in neuronal intracellular pH and to a rise in the pH_e. Secondly, bicarbonate independent alkaline shifts may arise from synaptic activity resulting in a flux of acid equivalents.

INTRODUCTION

Neural activity is accompanied by transmembrane ionic fluxes resulting in transient extracellular ionic shifts. It was not until the last 10 years that extracellular space (ECS) came to be recognized as a dynamically changing microenvironment for nerve cells, with a significant influence on the function of neurones and glia (for a review see refs. 43, 54, 56, 57). Impairment of the ionic homeostasis of the interstitial fluid therefore results in impairment of signal transmission. It is now accepted that besides the neuronal elements themselves, glial cells play an important role in extracellular K^+ homeostasis, as suggested originally by Hertz[32] and Orkand and colleagues[45]. The role of glia in the regulation of other extracellular ionic shifts and extracellular volume is less clear. However, recent findings also strongly support their role in the regulation of extracellular pH (pH_e) homeostasis (for a review see ref. 10).

During development astrocytes undergo marked morphological changes from flat polygonal cells to process-bearing cells and typical changes in glial intermediate filaments are reflected by the intensity of GFAP staining[21,23]. 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, hypertrophy and increases in GFAP staining[22,41]. Recent data suggest that immature astrocytes may not be able to perform their homeostatic function[33,48]. Consequently, it is of great interest to study how the ionic homeostasis and the relationship between neurones and glia differ during CNS development and reactive gliosis.

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This study deals with activity-related extracellular K^+ and pH_e changes in spinal cord of rats during postnatal development and after postnatal X-irradiation (PI)—a procedure which impaires gliogenesis and leads to astrogliosis. The early postnatal spinal cord X-irradiation is an experimental model developed and described by Gilmore[24,25]. X-irradiation effects in neonatal rat spinal cord include: reduction in oligodendrocytes; inhibition of myelin formation within the spinal cord, yet no extensive change in myelin formation in spinal roots; marked decreases in the number of neuroglial nuclei; hypertrophy of astrocytes, but no widespread alterations in spinal neurones[25]. Our experiments were designed to elucidate whether PI disrupts ionic homeostasis in spinal dorsal horn gray matter during postnatal development, and whether the proliferative and hypertrophied astrocytes are able to ensure ionic homeostasis. We expected that the glia, particularly astrocytes, could lose their functional ability after X-irradiation and this would result in greater activity-related $[K^+]_e$ changes and in large alkaline shifts in pH_e in spinal gray matter due to the inefficient glial membrane transport mechanisms. Using various membrane transport inhibitors we studied the possible mechanisms of the activity-related K^+ and pH_e changes during postnatal development and PI.

MATERIALS AND METHODS

Preparations

In the experiments performed on 2- 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 (in mM): NaCl 113.0, KCl 3.5, $CaCl_2$ 2.0, Na_2HPO_4 2.0, $NaHCO_3$ 28, 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). In HEPES-buffered saline $NaHCO_3$ was replaced by 20 mM HEPES and the solution was titrated to pH 7.3–7.35 with NaOH. Solutions with 20 mM $MgCl_2$ or 5 mM acetazolamide had reciprocally reduced Na^+ concentration.

Stimulation and Recording

Microelectrodes were inserted into the spinal cord from its dorsal surface. Recordings were made from lumbar segments L_4 or L_5. The dorsal root of the same segment was stimulated supramaximally (rectangular pulses of 5 V or less; duration 0.1 ms) with fine bipolar silver electrode. K^+ activity was recorded with double-barreled K^+-selective microelectrodes filled with a liquid ion-exchanger (Corning 477317) prepared by a procedure described previously[39]. In two experiments with amiloride the K^+-selective microelectrode was filled with a valinomycin ionophore (Fluka 60031). 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[15,60]. In principle, the reference channel was filled with 0.15 M NaCl solution while the pH-sensitive channel contained, in a siliconized tip, a 200–1000 μm column of liquid Hydrogen Ion Ionophore II-Cocktail A (Fluka). The backfilling solution was composed of: KH_2PO_4 40.0, NaOH 23.0 and NaCl 15.0 (mM, pH 7.0). Electrode sensitivity was tested in standard solutions the pH of which were 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 and the electrodes had a resistance of 700–1,800 MΩ. The electrical arrangements were the same as described for K^+-selective microelectrodes[39]. Each channel of a double-barrel microelectrode was connected to one input of a differential amplifier. Microelectrodes were inserted into the spinal cord using two micromanipulators.

X-irradiation

Rat pups, derived from different litters, were divided into control and X-irradiated groups. Animals 1 to 2 days old were X-irradiated according to Gilmore[24]. A single dose of about 40.0 Gy was administered with a X-Ray Therapy Unit (Phillips RT 100) under the following parameters: 50 KVP; 10 mA; 10 cm FSD; total dose, 41.1 Gy, dose rate, 7.733 Gy/min; added filtration 0.3 mm aluminium; half-value layer (HVL), 0.2 mm aluminium. Prior to irradiation calibration was made in a cylindrical ionization chamber. The irradiated area was restricted to spinal cord lumbosacral region demarcated by covering the animal with a protective lead shield, 0.3 mm in thickness containing a slit measuring 5 mm anteroposteriorly by 10 mm transversely[24].

Immunostaining

The postnatal maturation of astrocytes and their reaction to X-irradiation was analyzed by immunostaining. The appearence and distribution of glial fibrillary acidic protein (GFAP) immunoreactivity was shown light microscopically. Animals 3 to 14 days old were anesthetized with Nembutal and perfused transcardially with 0.9% saline followed by a mixture of 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.25). Spinal cords were dissected, immersed into the sucrose (30%) PB and kept in a refrigerator before cutting on a cryostat. Cryostat sections of lumbal spinal cord were processed for immunohistochemistry using Vectastain ABC Kit (Vector Labs., Inc.). The procedure started with incubation of sections for 20 min with diluted normal blocking serum which was prepared from the species in which the secondary antibody was made (goat serum) to suppress non-specific immunoreactivity. Sections were then incubated for 60 min with primary antiserum to GFAP (anti-GFAP, rabbit, SIGMA). The primary antiserum was diluted 1:200. As the second antibody, biotinylated rabbit IgG and immunoperoxidase procedure was used. Sections were incubated for 30 min with Vectastain ABC Kit, which contains Avidin DH, a glycoprotein with extraordinarily high affinity for biotin, and biotinylated horseradish peroxidase H. The immune-complex molecule was visualized by 3,3′-diaminobenzidine tetrachloride (DAB, Sigma) reaction.

Applications of drugs

The drugs were dissolved and applied onto the spinal cord in Ringer's solution. We used acetazolamide (Ciech); amiloride (Merck, Sharp Dohme); SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid), GABA and picrotoxin (Sigma); $MgCl_2$ and $BaCl_2$ (BDH Chemicals Ltd.).

Statistical Analysis

Results of each experiment were expressed as the means ± S.E.M. Statistical analysis of the differences between groups was evaluated by Student's t-test. Values of $P < 0.05$ were considered significant.

RESULTS

Immunohistochemical findings

Typical development of GFAP-positive reaction was observed during the first 14 days postnatally in non-irradiated (control) pups. In 2- to 6-day-old pups the membrane limitans was densely stained as well as radial glia from subpial zone inward through white

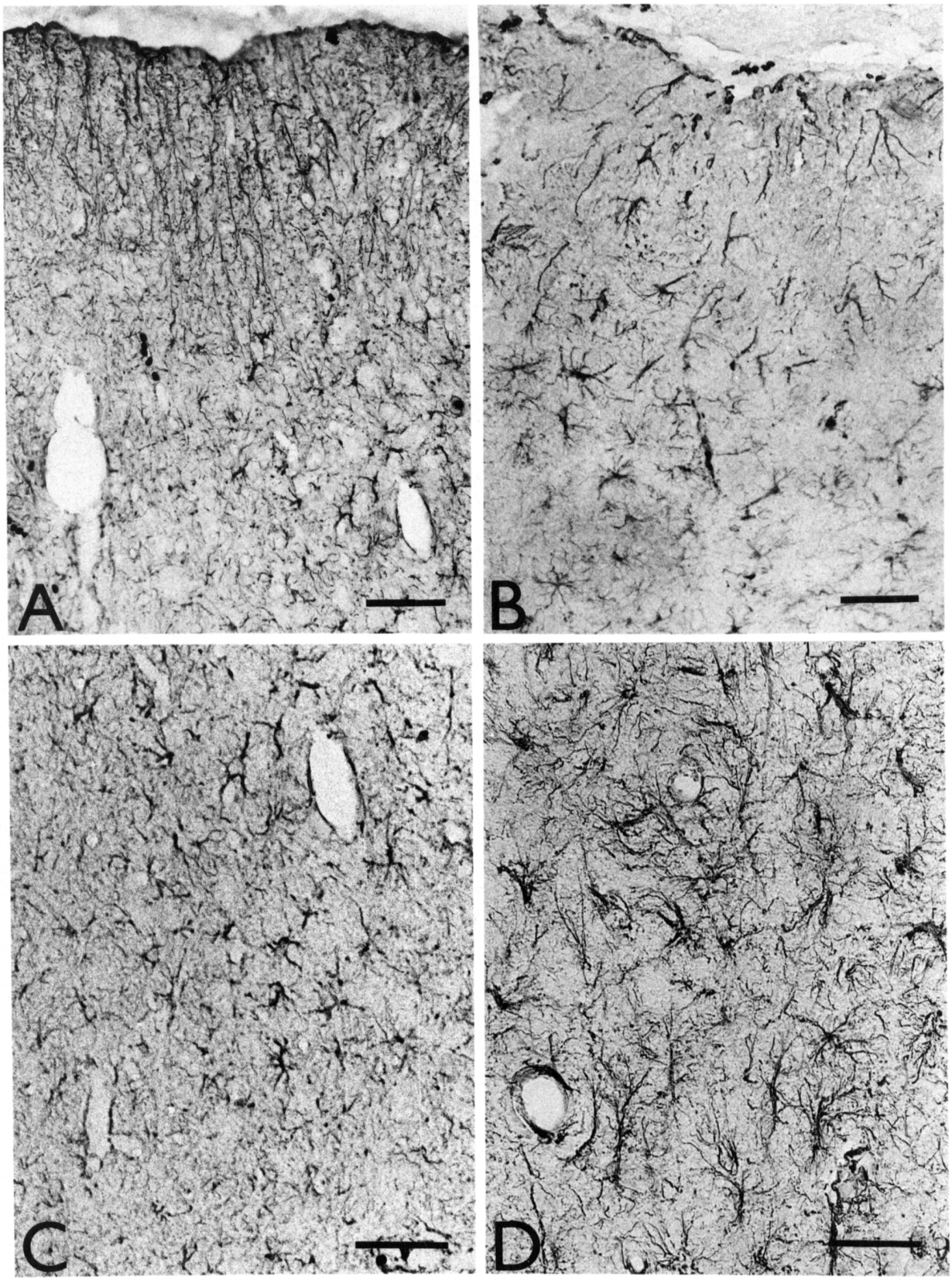

Fig. 1. Astrocytes revealed by GFAP immunostaining in spinal dorsal horns of 12-day-old rats. A: radial glia in the white matter and C: fibrous astrocytes in the gray matter of the control (non-irradiated rat). B and D: effect of X-irradiation of the spinal cord at the first day postnatally (PI). Note the reduction of GFAP immunoreactivity in the white matter (B) and astrogliosis with increased GFAP reaction in the gray matter PI (D). Bars = 50 μm.

matter. In gray matter, the small densely stained somata with very short processes were seen (not shown). In 10- to 14-day-old pups, the typical radial glia from the subpial zone inwards was still densely stained (Fig. 1A) and astrocytes in the gray matter were stained more intensively than in 2 to 6-day-old pups; they showed larger somata and longer processes (Fig. 1C). On the contrary, in 10- to 14-day-old pups PI GFAP staining of radial glia in white matter was disrupted (Fig. 1B). GFAP staining of astrocytes in gray matter revealed a typical astrogliosis—astrocytes had unusually large somata and thick, long processes, giving the impression of an increased number of proliferative and hypertrophied astrocytes (Fig. 1D).

K^+ and pH_e resting level

In unstimulated pups the $[K^+]_e$ baseline in the lower dorsal horn at a depth of 250–350 μm from the dorsal surface was elevated and the pH_e baseline was more acid than that in Ringer's solution (3.5 mM K^+, pH 7.3–7.35). The $[K^+]_e$ and pH_e in the early postnatal period, i.e. in 3- to 6-day-old pups was 3.91 ± 0.12 mM K^+ ($n = 14$) and 7.19 ± 0.01 pH units ($n = 14$); while in 10- to 14-day-old pups it was 4.35 ± 0.15 mM K^+ ($n = 10$) and 7.11 ± 0.01 pH units ($n = 10$)[33]. In 10- to 14-day-old pups PI the measurements of $[K^+]_e$ and pH_e baseline revealed their return towards the early postnatal values, i.e. K^+ significantly decreased to 3.83 ± 0.16 mM ($n = 9$, $P < 0.005$) and pH_e significantly increased to 7.18 ± 0.02 ($n = 7$, $P < 0.05$).

Stimulation-evoked K^+ and pH_e changes

Electrical stimulation of a dorsal root produces an increase in $[K^+]_e$ and alkaline-acid shifts in pH_e baseline in the corresponding spinal cord segments of adult and immature rats[33,51,60]. When repetitive stimulation continues for more than 6–8 s no further increase in $[K^+]_e$ is found because a steady state is established, which is a result of concurrent release and clearance of K^+ (Fig. 2). This 'ceiling' level[31,40] in the CNS of adult rats is achieved at a stimulation frequency of 30–100 Hz (Fig. 2—90 days) and is only broken through by pathological events, e.g. epileptic activity, anoxia, spreading depression, application of convulsive drugs

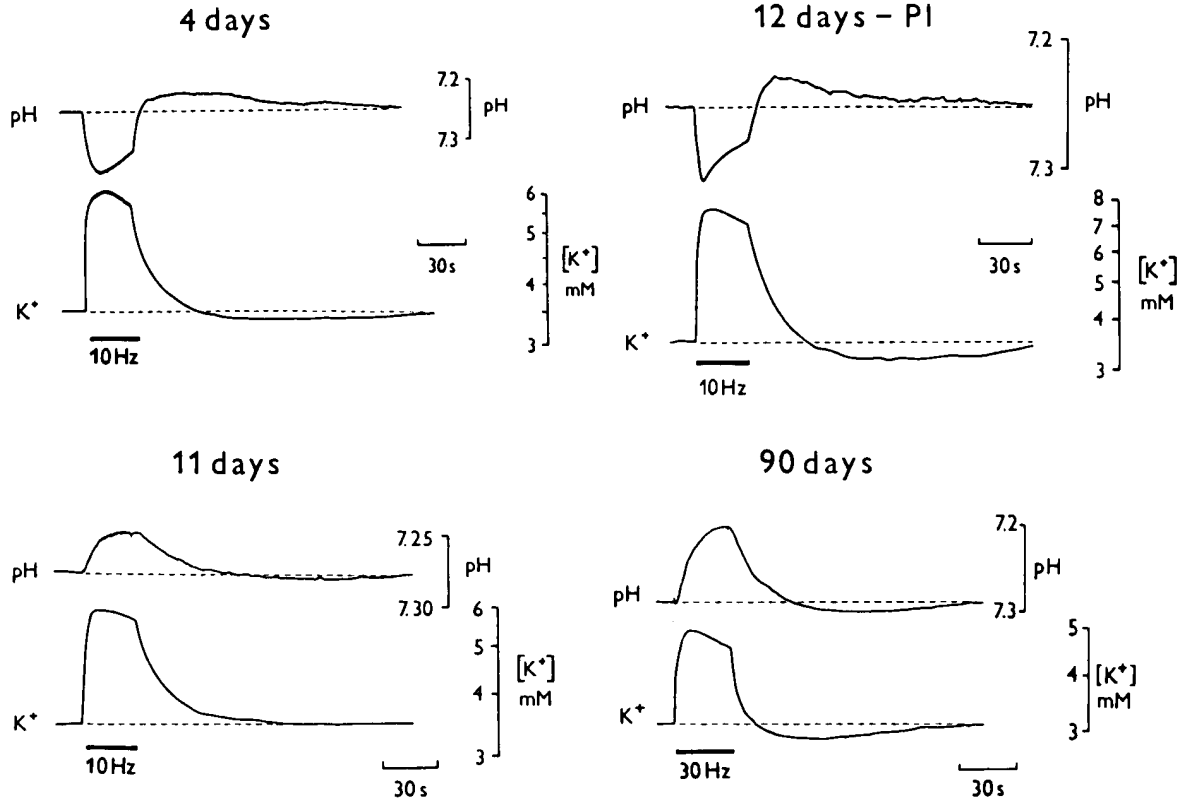

Fig. 2. Stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of non-irradiated rats 4, 11 and 90 days old and in 12-day-old rats PI. Note that the stimulation of the dorsal root at a frequency of 10 Hz evoked an alkaline shift in the 4-day-old pup, which was accompanied by an increase in the $[K^+]_e$ ceiling level; when stimulation was discontinued the poststimulation acid shift of smaller amplitude appeared, which was accompanied by a K^+-undershoot. Similar changes were found in 12-day-old animals PI. In the 11-day-old and 90-day-old (adult) rat the stimulation evoked a dominating acid shift and the $[K^+]_e$ ceiling level decreased.

(for a review see refs. 54, 57). In spinal cord, the ceiling level was higher in immature rats and it has been suggested that K^+ homeostasis is impaired due to incomplete gliogenesis[33]. The ceiling level in 2- to 14-day-old rats was already attained at a stimulation frequency of 10 Hz. The maximal changes in $[K^+]_e$ progressively decreased with the animals age. In 3- to 6-day-old non-irradiated rats, maximal changes in $[K^+]_e$ at a stimulation frequency of 10 Hz were 4.03 ± 0.24 mM ($n = 30$) ranging from 2.5 to 6.5 mM (Fig. 2—4 days). In 10- to 14-day-old rats the maximal changes in $[K^+]_e$ were 1.95 ± 0.12 ($n = 30$) ranging from 0.6 to 3.0 mM (Fig. 2–11 days).

Fig. 2 further shows that in 3- to 6-day-old pups, the stimulation produced an alkaline shift of 0.048 ± 0.004 pH units ($n = 32$) ranging from 0.02 to 0.15, which was followed by a smaller poststimulation acid shift ranging from 0.02 to 0.07 pH units. In 10- to 14-day-old rats, however, the stimulation evoked an acid shift of 0.035 ± 0.003 pH units ($n = 40$) ranging from 0.02 to 0.1, which was in some animals preceded by a scarcely discernible alkaline shift. In adult rats, stimulation produces the dominating acid shift of 0.1–0.2 pH units[60], which is preceded by a scarcely discernible initial alkaline shift, and when stimulation is discontinued the acid shift is followed by a poststimulation alkaline undershoot (Fig. 2—90 days; see also ref. 60).

In 10- to 14-day-old rats PI the stimulation-evoked $[K^+]_e$ and pH_e changes still resembled those found in early postnatal days, i.e. in animals 2 to 6 days old. The stimulation at frequency 10 Hz increased $[K^+]_e$ by 4.03 ± 0.25 mM ($n = 12$) ranging from 2.5 to 5.0 mM, and there was a typical dominant stimulation-evoked alkaline shift of 0.053 ± 0.008 pH units ($n = 12$) ranging from 0.02 to 0.11 pH units, which was followed by a delayed smaller acid shift of 0.02–0.05 pH units when stimulation was discontinued (Fig. 2—12 days PI). These findings show that postnatal X-irradiation impairs normal development of extracellular K^+ and pH_e homeostasis in the rat spinal cord.

Effects of Mg^{2+} and acetazolamide

Application of 20 mM $MgCl_2$ reversibly blocked the alkaline shifts in 3- to 6-day-old rats[33] and in animals PI (Fig. 3, $n = 7$). The acid shift in control animals 10- to 14-day-old was not affected by Mg^{2+}[33]. Since Mg^{2+} is known to block Ca^{2+}-dependent presynaptic transmitter release, this shows that the alkaline shift in immature animals and in animals PI is of synaptic origin. The application of Mg^{2+} also lowered the $[K^+]_e$ changes by 80–90%, suggesting that only about 10–20% of stimulation-evoked K^+ release could be presynaptic, i.e. from activated primary afferent fibres, as is the

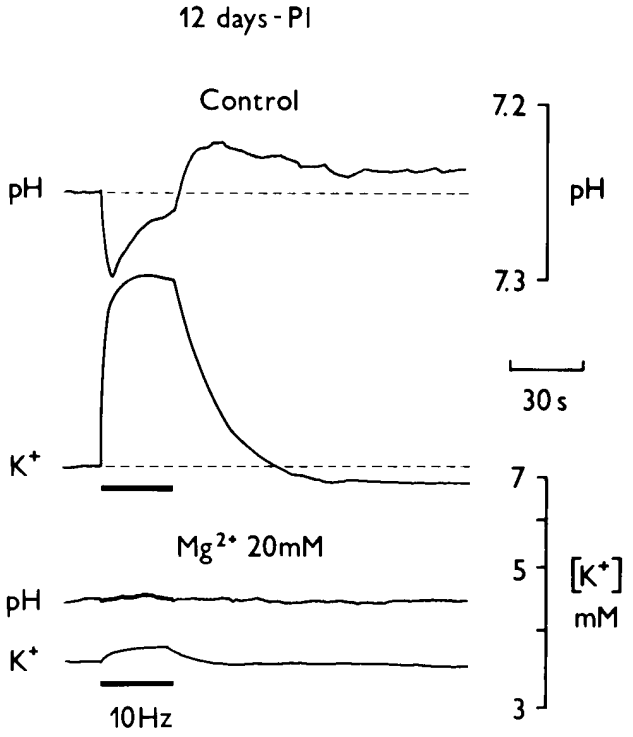

Fig. 3. Effect of 20 mM Mg^{2+} on stimulation (10 Hz)-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 12-day-old rats PI. Note the disappearance of the alkaline shift, and decrease of $[K^+]_e$ changes.

case in adult animals[62]. Our results therefore suggest that the alkaline shifts in pH_e are not related to gliogenesis, while the acid shifts are.

The alkaline shifts in immature rats[33] and in animals PI were either not changed or slightly enhanced (in 3 experiments out of 6) by the carbonic anhydrase (CA) inhibitor acetazolamide in 5–500 μM concentrations. The alkaline response in the presence of acetazolamide increased by $26.4 \pm 15.9\%$ ($n = 6$). The acetazolamide blocked the poststimulation acid shift in 2- to 6-day-old rats and in rats PI, similarly to our previous finding that acetazolamide decreases the amplitude of the acid shift in 10- to 14-day-old rats[33]. The enzyme CA which catalyzes the conversion of CO_2 and water to HCO_3^- and H^+, is mainly located in glia and myelin[6,49], but the presence of extracellular CA has also been reported[9,34]. Our results suggest that there is little or no extracellular CA present during the early postnatal period and in animals PI.

Effects of inhibitors of membrane transport systems

It has been shown that acid extrusion in CNS neurons and glia involves amiloride-sensitive Na^+/H^+ exchange, SITS-sensitive Cl^-/HCO_3^- or $Na^+/Cl^-/H^+/HCO_3^-$ exchange as well as Na^+-HCO_3^- cotransport (for review see refs. 10, 55, 57, 63, 65). We studied the

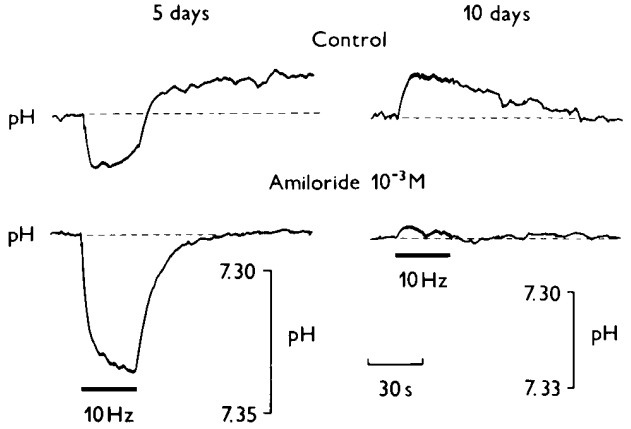

Fig. 4. Effect of amiloride (10^{-3} M) on stimulation (10 Hz)-evoked pH_e changes in the spinal dorsal horn of 5- and 10-day-old rats. Note the enhancement of the alkaline shift and diminution of the post-stimulation acid shift in 5-day-old rats, and decrease of the acid shift in 10-day-old rats.

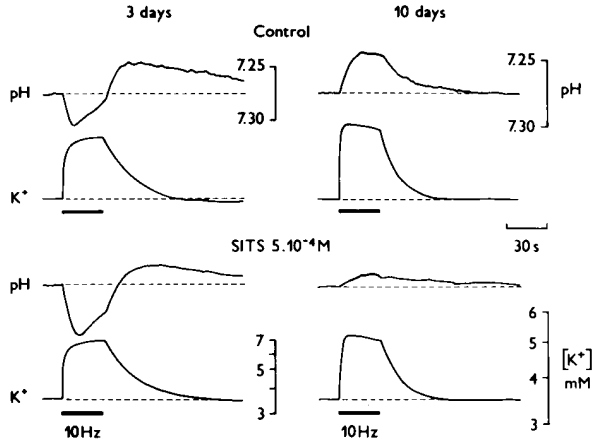

Fig. 6. Effect of SITS (5.10^{-4} M) on stimulation (10 Hz)-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 3- and 10-day-old rats. Note the enhancement of the alkaline shift and diminution ot the poststimulation acid shift in 3-day-old rats, and the decrease in the acid shift in 10-day-old rats.

effect of inhibitors of membrane transport processes on the activity-related alkaline shift, presumably of neuronal origin, and on the acid shift which was suggested to be related to gliogenesis[33].

Effect of amiloride. The application of amiloride in concentrations of 3 mM enhanced the alkaline and blocked the acid shifts evoked by stimulation. Typical results are shown in Fig. 4 and Fig. 5. In 3- to 6-day-old rats and in rats PI amiloride enhanced alkaline shifts by $105.7 \pm 34.4\%$ ($n = 8$) and blocked the poststimulation acid shift. Amiloride decreased the stimulation-evoked acid shift in 10- to 14-day-old rats by $62.5 \pm 4.7\%$ ($n = 8$, Fig. 4). We could not record the effect of amiloride on stimulation-evoked $[K^+]_e$ changes in all

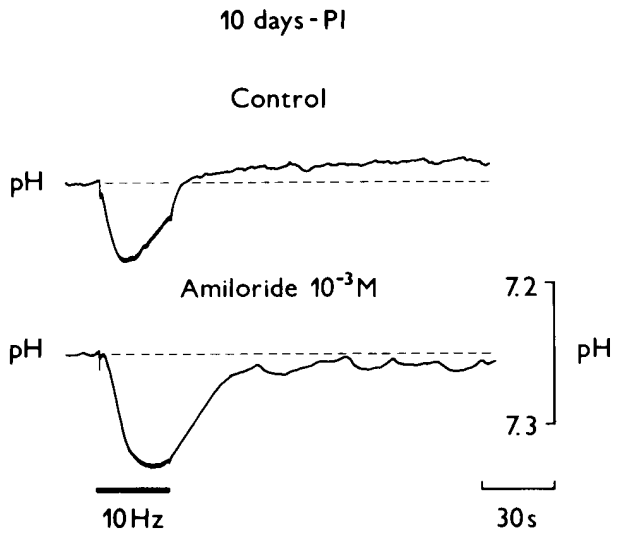

Fig. 5. Effect of amiloride (10^{-3} M) on stimulation (10 Hz)-evoked pH_e changes in the spinal dorsal horn of 10-day-old rats PI. Note the enhancement of the alkaline shift and diminution of the poststimulation acid shift.

our experiments to test its possible effect on impulse transmission, since the K^+-selective microelectrodes (Corning 477317) are sensitive to amiloride[60]. However, no effect on $[K^+]_e$ was found in two experiments in which we used K^+-selective microelectrodes with a valinomycine ionophore.

Effect of SITS. Application of SITS in concentrations of 0.5 mM had a similar effect on stimulation-evoked pH shifts as amiloride. In 3- to 6-day-old rats and in rats PI SITS enhanced the alkaline shift by $141.7 \pm 24.0\%$ ($n = 4$), while it decreased the poststimulation acid shift (Fig. 6 and Fig. 7). In 10- to 14-day-old rats the dominating acid shift was decreased by $81.7 \pm 5.2\%$ ($n = 6$). In concentrations of 0.5 mM SITS does not significantly affect changes in $[K^+]_e$, showing that the observed effects were not due to general changes in excitability.

Effect of Ba^{2+}. In the presence of Ba^{2+} the sensitivity of the glial membrane to changes in K^+ is reduced, because of competition between K^+ and Ba^{2+} for K^+ channels. Indeed, in control rats 10 to 14 days old the addition of 0.5 mM Ba^{2+} resulted in dramatic enhancement of the K^+ ceiling level up to 6.63 ± 0.75 mM ($n = 4$) compared with an avarage control response prior to application of Ba^{2+} 2.33 ± 0.27 mM (Fig. 9), presumably because the K^+ buffering by glia was blocked. The addition of Ba^{2+} had no effect on the already higher K^+ ceiling level in 3- to 6 day-old rats (Fig. 8, 4.48 ± 0.53, $n = 6$) and in rats PI (4.32 ± 0.10, $n = 4$), apparently due to incomplete gliogenesis (Fig. 10).

Glial cells appear to extrude acid in response to membrane depolarization due to activity-related rises in $[K^+]_e$[12,13]. In 3- to 6-day-old rats application of Ba^{2+}

enhanced the stimulation-evoked alkaline shifts by 96.6 ± 21.2% ($n = 6$; Fig. 8). In 10- to 14-day-old rats Ba^{2+} decreased the acid shifts by 61.1 ± 3.5% ($n = 6$) or even reversed the acid shift to alkaline (Fig. 9). However, in 10- to 14-day-old rats PI Ba^{2+} had no effect on alkaline shifts (Fig. 10). These results suggest that the stimulation-evoked acid shift in pH_e is related to depolarization of glial cell membranes by elevated K^+.

Effect of GABA, picrotoxin and HCO_3^--free media

It has been shown that the GABA-gated anion channel has a significant permeability to HCO_3^- (refs. 5, 35, 36, 37). Since the electrochemical gradient for HCO_3^- is outward, release of GABA can evoke a HCO_3^--dependent fall in intracellular pH of neurones and a concomitant rise in pH_e. In our experiments superfusion of spinal cords of 3- to 14-day-old control rats as well as X-irradiated rats, with GABA (10^{-3} M), elicited extracellular transient alkaline shift in the pH_e baseline by 0.057 ± 0.009 pH units ($n = 6$, Fig. 11A), which was blocked by picrotoxin (not shown). Regardless of continuous GABA application, the alkaline shift in pH_e lasted only several minutes and then pH_e recovered and rebound to acidification, presumably due to receptor desensitization or due to one of the

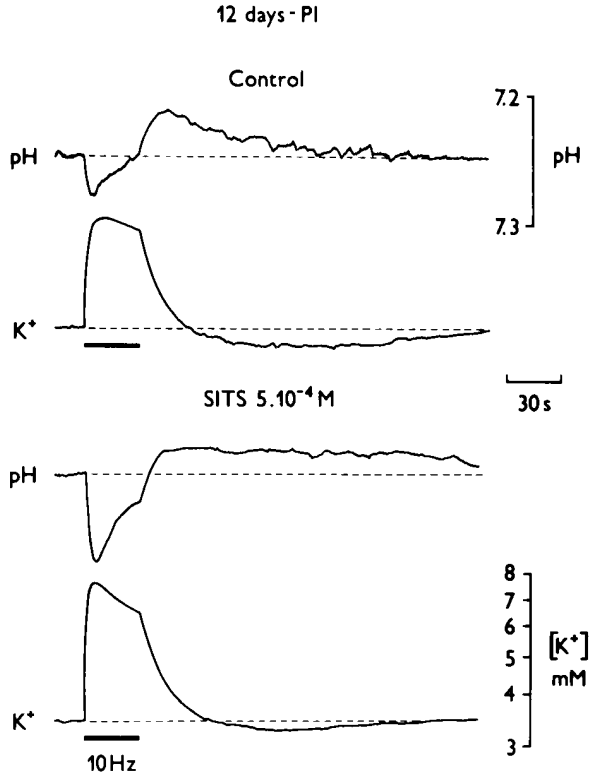

Fig. 7. Effect of SITS (5.10^{-4} M) on stimulation (10 Hz)-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 12-day-old rats PI. Note the increase of the alkaline shift and decrease of the poststimulation acid.

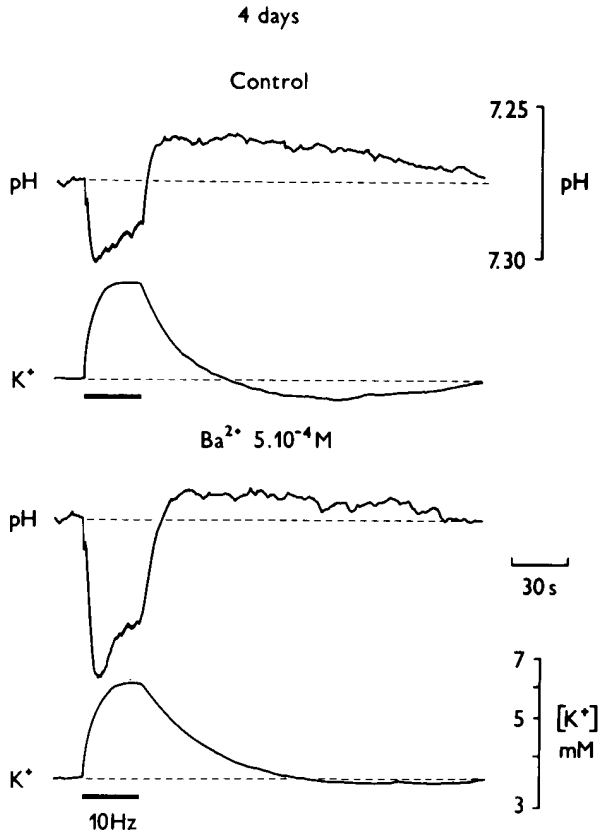

Fig. 8. Effect of Ba^{2+} (5.10^{-4} M) on pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 4-day-old rats. Note the enhancement of the stimulation-evoked alkaline shift and the decrease in poststimulation acid shift. The $[K^+]_e$ ceiling level was not changed.

acid extrusion mechanisms, which take part in buffering the alkaline shift. Picrotoxin in concentrations 10^{-7}–10^{-6}M had no effect on $[K^+]_e$ and the pH_e baseline; however it depressed the stimulation-evoked alkaline shifts in pH_e by 53.8 ± 2.08% ($n = 4$, Fig. 11B). It has been found in the isolated frog spinal cord that superfusion with GABA in concentrations 10^{-3}–10^{-2} M increases $[K^+]_e$[53]. Indeed, the alkaline shifts evoked in our experiments by superfusion of the spinal cord with GABA were accompanied by an increase in $[K^+]_e$ (Fig. 11A). However, the superfusion of spinal cord with Ringer solution containing elevated K^+ produced in 3- to 14-day-old controls, as well as X-irradiated pups, an acid and not an alkaline shift in the pH_e baseline (Fig. 11C).

In nominally HCO_3^--free solution, buffered with HEPES, GABA evoked no alkaline shift, instead we observed a slow acid shift. In most cases, the change from a solution buffered with HCO_3^- to a nominally HCO_3^--free solutions buffered with 20 mM HEPES, resulted in transient alkaline shift in pH_e baseline by about 0.1 pH units. However, in 10 min the pH_e baseline in the HEPES-buffered solution returned to the pH_e baseline in the HCO_3^- buffered solution. The

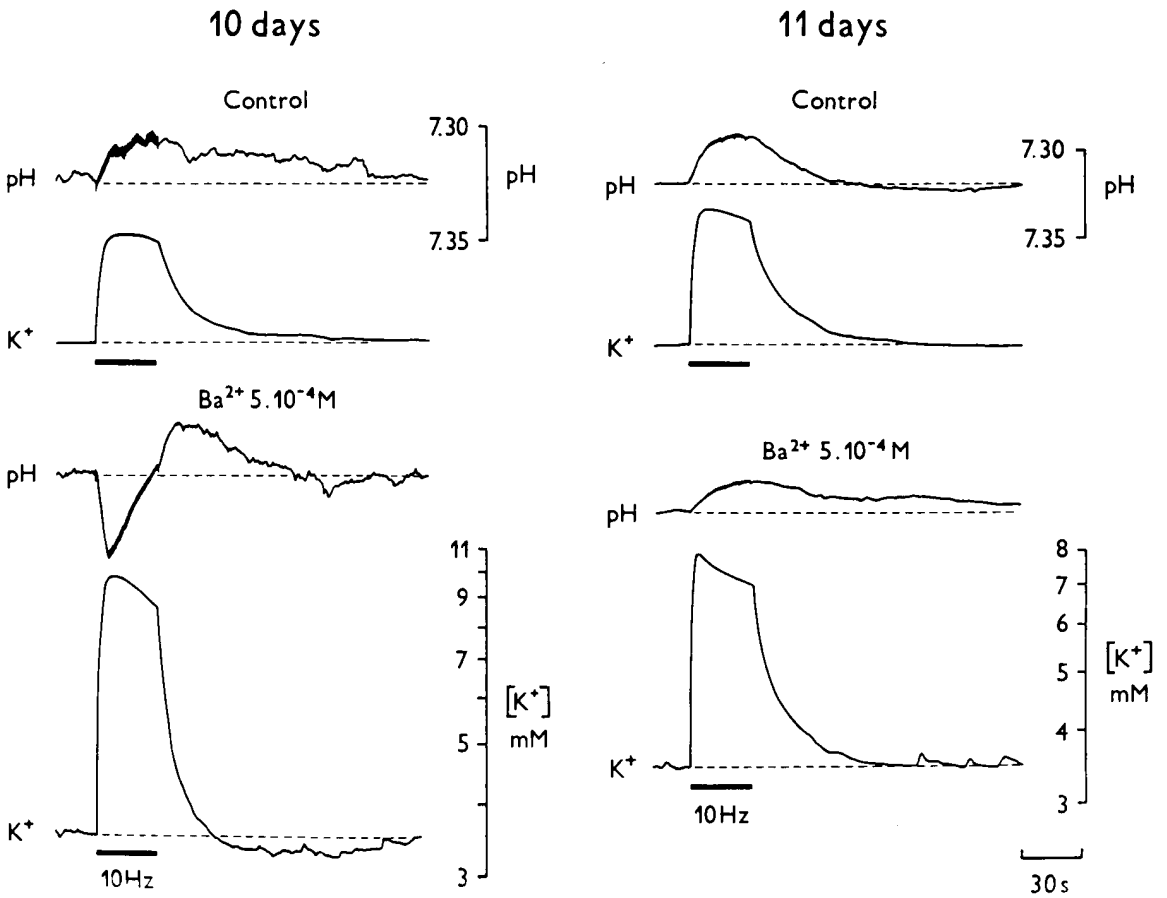

Fig. 9. Effect of Ba^{2+} (5.10^{-4} M) on pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 10- and 11-day-old rats. Note the reversal of acid shift into alkaline shift in 10-day old rats and decrease in acid shift in 11-day-old rats. Ba^{2+} enhanced the $[K^+]_e$ ceiling level.

stimulation evoked alkaline shifts in 2- to 6-day-old rats and in rats PI were enhanced in the HEPES-buffered solution by 217.1 ± 38.2% ($n = 4$).

These experiments suggest that gating of $GABA_A$ channels in immature 3- to 14-day-old rats as well as in rats PI can give rise to an HCO_3^- efflux which may partially explain the pH_e alkaline shifts observed in the rat dorsal horn during stimulation of an afferent input. However, our results indicate that the observed alkaline shifts can also be mediated by an HCO_3^--independent mechanism.

DISCUSSION

K^+ homeostasis

Active neurones as well as primary afferent fibres lose K^+ and induce a change in the ionic composition of the ECS (for reviews see refs. 54, 56, 57). 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. K^+ homeostasis in CNS is ensured by two main mechanisms: (1) activation of the Na/K pump in neurones and glia, and (2) glial cell KCl uptake and/or K^+ spatial buffering of the rise in $[K^+]_e$[54,57,65]. The conclusion that K^+ is cleared by neurones by means of active transport is strongly supported in spinal cord, since there is a poststimulation decrease below resting level, the so-called 'K^+-undershoot' (Fig. 2), which can be blocked by inhibitors of the Na/K pump (e.g. ouabain), anoxia, ischemia or anesthetic drugs[31,40,58]. However, glial cells in spinal cord may also significantly contribute to activity-related clearance of K^+ increase in ECS. There is evidence in the spinal cord that K^+ clearance by glia includes K^+ spatial buffering[59].

In 3- to 6-day-old rats and in rats PI K^+ and pH_e homeostasis is impaired[33,64], but there are no apparent changes in neuronal Na/K pump activity (see Fig. 2–normal K^+-undershoot) and the increase in $[K^+]_e$ is as fast as in adult rats[60]. Similarly, larger stimulation-evoked changes in $[K^+]_e$ were found in immature optic nerve[16,47,48] and in cerebral cortex[29,42]. The changes are larger in spite of the facts that the size of the ECS in immature nervous tissues could be larger and that the stimulation of the afferent input does not cause ECS shrinkage which can aggravate the ionic changes[47].

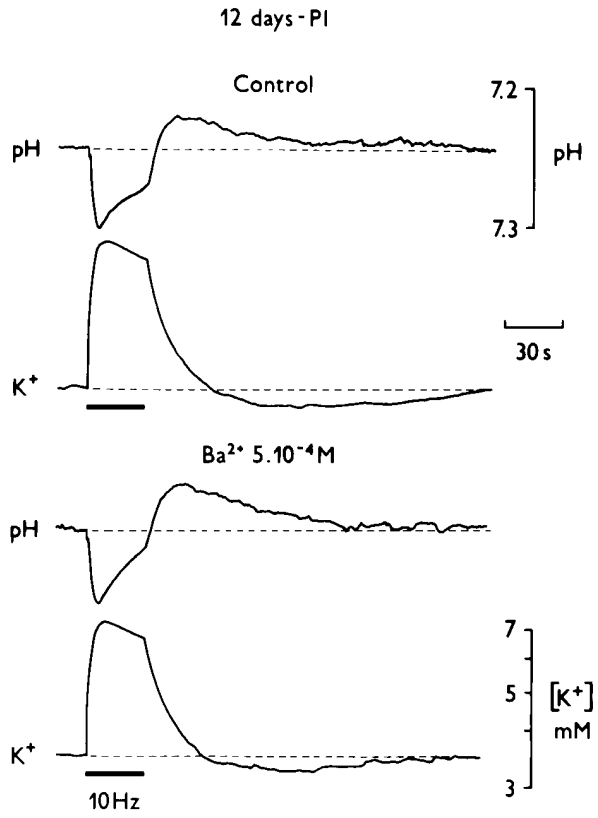

Fig. 10. Effect of Ba^{2+} (5.10^{-4} M) on stimulation-evoked pH_e and $[K^+]_e$ changes in the spinal dorsal horn of 12-day-old rats PI. Ba^{2+} had no effect on pH_e and $[K^+]_e$ changes.

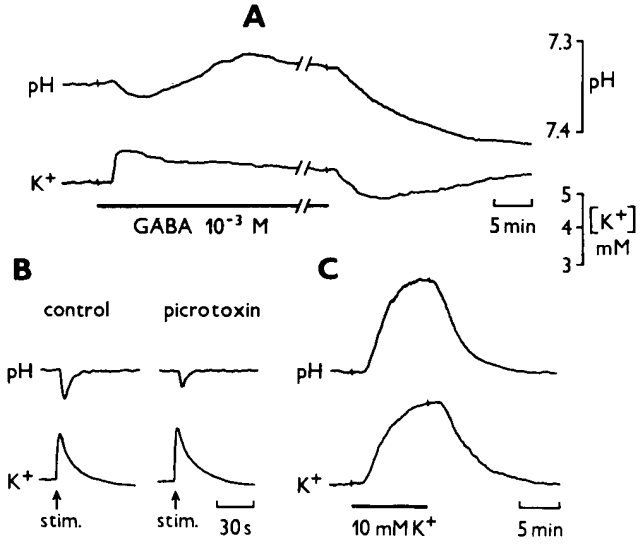

Fig. 11. A: effect of GABA (1 mM) on pH_e and $[K^+]_e$ baseline in the isolated spinal cord of 4-day-old rats. Note the alkaline shift and increase in $[K^+]_e$ during application of GABA. B: stimulation of the dorsal root with a single electrical pulse evoked an alkaline shift in 5-day-old rats, which was accompanied by an increase in $[K^+]_e$. Application of picrotoxin (10^{-6} M) decreased the alkaline shift. C: simultaneous pH_e and $[K^+]_e$ records in the dorsal horn during perfusion of the spinal cord with a Ringer's solution containing 10 mM K^+. Records in A and B are from the same experiment.

The K^+ ceiling level gradually decreased with the animal's age, being comparable in 12- to 14-day-old pups with the K^+ ceiling level in adult rats[51]. A large increase in the K^+ ceiling level in 10- to 14-day-old control animals was found after blockade of glial cells' K^+ channel permeability by Ba^{2+} (see Fig. 9), while there was no further increase in ceiling level in immature rats and in rats PI (Figs. 8 and 10). The maturation of astrocytes, oligodendrocytes and myelinization[16,26,27,50,68], as well as CA activity[17], density of K^+ channels and Na^+/K^+ ATPase sensitivity to elevated $[K^+]_e$[28], occurs in the first 8–10 days postnatally. However, we can not exclude the possibility that the rate of K^+ release from neurones as well as the properties, distribution and density of Na^+, K^+ and Cl^- channels in neurones and axons can also change during development and that X-irradiation spares neurones entirely (for further discussion see refs. 33, 64, 65). Nevertheless, the observed differences in K^+ ceiling level suggest that immature glia in the first 8–10 days postnatally and proliferative glia after PI are not able to control K^+ homeostasis.

pH_e homeostasis

Concomitantly with an increase in $[K^+]_e$, any neuronal activity also results in a transient change in ECS acid-base balance[10,56,57]. In adult rat spinal cords the activity-related pH_e changes have a typical time-course: the initial small alkaline shift which has the same latency as the increase in $[K^+]_e$ is followed by the dominating acid shift during the stimulation and by a poststimulation alkaline undershoot[55,60] (Fig. 2). 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). Both in 3- to 6-day-old pups and in pups PI, the pH_e baseline was more alkaline than that in non-irradiated animals 10 to 14 days old, suggesting that the maturation of glia results in an acid shift of the pH_e baseline.

It is well documented that X-irradiation of the spinal cord of 1- to 2-day-old pups disrupts gliogenesis. At 9–30 days of age PI there is a severe reduction in the number of glial cells, a concurrent state of hypomyelination and astrogliosis in lumbosacral spinal cord[24,25]. Despite that, axons appear to be normal and continue to grow normally in the glial-depleted region[4,30,46]. However, we cannot entirely exclude an PI effect on spinal neurones. Alkaline shifts evoked by stimulation of the afferent input in rats PI closely resembled those found in the early postnatal period suggesting that X-irradiation impaired pH_e homeostasis. X-irradiation blocked the acid shifts which are closely related to

spinal cord postnatal development, presumably primarily to gliogenesis[33]. While it is evident that astrocytes undergo some degree of functional maturation in the first 10 days postnatally (Fig. 1), it is also evident that they are not yet fully mature by day 14, since adult astrocytes are only lightly stained for GFAP and have very long fine processes[21]. The pH_e changes (acid shifts) evoked in 10- to 14-day-old rats by stimulation were qualitatively similar to, but about 2–4 times smaller than those in adult rats (see Fig. 2). It has been suggested that neurones also extrude acid and therefore the acid shift in adult rats may be the result of both neuronal and glial acid extrusion mechanisms[38,60]. In adult rats not only the alkaline, but also the acid shifts can be blocked by about 65–70% by Ca^{2+}-channel blockers, and they can be partly blocked by La^{3+}, an H^+ channel blocker[60]. We can therefore assume that in adult animals the stimulation-evoked acid shifts in pH_e are due to acid extrusion from both neurones as well as glial cells. This might not be the case in 10- to 14-day-old rats in which the acid shift is not affected by Ca^{2+} channel blockers, and is therefore apparently largely of glial cell origin.

Mechanisms of alkaline-acid shifts in pH_e

Current research suggests that activity-related alkaline shifts may be due to: (1) channel-mediated fluxes of bicarbonate, (2) channel-mediated acid influx into neurones, (3) inhibition of glycolytic acid source, and (4) extracellular volume shrinkage (for review see refs. 10, 57). The Ca^{2+} channel blockers (Mn^{2+}, Mg^{2+}) diminished the alkaline shifts, suggesting that the activity-related alkaline shifts are related to synaptic activity[33,61] (see Fig. 3). The contribution of the stimulation-enhanced blood flow and consequent increased washout of CO_2 to the alkaline shift was suggested by Urbanics and colleagues[63]. This possibility was, however, ruled out in experiments in vitro[11,15,17,20,33,38,44,61,66]. Alkaline shifts were found to persist in HCO_3^- free solutions[11,14], which rules out the possibility that shrinkage of the extracellular space associated with neuronal activity[52] would concentrate extracellular HCO_3^-. Alkaline shifts in our experiments were found to be increased and/or unmasked by any blockade of stimulation-evoked acid shifts, e.g. by amiloride, SITS or DIDS. Amiloride and SITS enhanced the alkaline and blocked acid shifts also in slices of the rat hippocampus[66]. Similarly to the findings in the isolated frog spinal cord, the alkaline shifts in immature and in X-irradiated rats were enhanced in HEPES-buffered saline, and in some of our experiments when activity of CA was blocked by acetazolamide[55]. These findings suggest that the extracellular bicarbonate serves to buffer the pH_e changes. Recently, evidence has been presented that the extracellular CA plays an important role in stimulation-evoked alkaline–acid changes in rat hippocampal slices[9,34]. The large alkaline shifts in 3- to 6-day-old immature rats and in rats PI and the modest effect of acetazolamide, may indicate that the extracellular CA activity in the neonate rats is low.

Alkaline shifts related to neuronal activity have been described in many studies (for review see refs. 10, 57). Recently it has been suggested that alkaline shifts in turtle cerebellum are due to channel-mediated flux of acid equivalents, e.g. efflux of HCO_3^- through $GABA_A$-gated Cl^- channels[7]. In our experiments the superfusion of spinal cord with GABA (10^{-3} M) in the presence of HCO_3^- elicited an extracellular alkaline shift in control 3- to 14-day-old pups as well as in pups PI (Fig. 11A), which was blocked by picrotoxin in concentrations 10^{-7}–10^{-6} M (in this concentration picrotoxin had no effect on K^+ and pH_e resting levels). This suggests that Cl^- channels opened by GABA in the spinal cord give rise to an HCO_3^- efflux which, as is the case in the CNS[7,8] and in muscle fibres[33,35], increase the pH_e. It has been found that GABA in concentrations 10^{-3}–10^{-2} M increases $[K^+]_e$ in the frog spinal cord[53]. Indeed, the alkaline shift evoked by superfusion of GABA in 3- to 14-day-old pups was accompanied by an increase in $[K^+]_e$ (Fig. 11A). This increase in $[K^+]_e$, however, cannot explain the alkaline shift, since our experiments with superfusion of the spinal cord with Ringer solution containing elevated K^+ demonstrated that the increase in $[K^+]_e$ is accompanied by an acid shift in pH_e baseline (Fig. 11C). It should be stressed here that GABA-evoked alkaline shifts are not related to gliogenesis, since they were well developed in the early postnatal period and were not affected by X-irradiation. However, it is evident that stimulation-evoked alkaline shifts are not solely due to bicarbonate efflux. Activity-related alkaline shifts could also be generated by a bicarbonate-independent mechanism related to excitatory synaptic transmission[9, 14]. Excitation of various cationic and anionic channels, either ligand- or voltage-gated, can result in acid fluxes through these channels and remain to be tested.

In the spinal cord of 3- to 14-day-old rats Ba^{2+}, which blocks K^+ channel permeability in glial cells, enhanced the K^+ ceiling level, blocked the stimulation-evoked acid shift, and enhanced or unmasked the alkaline shift (Figs. 8, 9). It has been suggested that large evoked extracellular K^+ increase in the brain observed in Ba^{2+} -treated preparations in vivo is due to the impairment of passive and active K^+

clearance by glial cells[67]. Ballanyi and colleagues[3] found that Ba^{2+} blocked the stimulation-evoked increase in $[K^+]_i$ in the glia and their depolarization in guinea-pig olfactory cortex slices. With superfusion of the rat cortex with Ba^{2+}, the initial alkaline shift was also enhanced[13]. Mammalian cortical astrocytes depolarize and become more alkaline during stimulation[13]. Superfusion of the cortical surface with Ba^{2+} caused hyperpolarization of the glia during stimulation and completely abolished the intraglial alkaline shift. It has therefore been suggested that the mechanism of the stimulation-evoked intraglial alkaline shift involves extrusion of acid. The sensitivity to Ba^{2+} indicates that it is triggered by glial membrane depolarization and that glial pH_i is modulated by the level of local neuronal activity. If the HCO_3^- is a counterion to K^+ during glial cell depolarization, the block of K^+ channels with Ba^{2+} would result in a block of the pH_e acid shift. Electrogenic Na^+-HCO_3^- cotransport could be responsible for the depolarization-induced alkaline shifts of glial cells[1, 2, 18] and contribute to the observed stimulation-evoked acid shifts in pH_e. Blocking any acid extrusion system in glial cells would therefore enhance any extracellular alkaline shift as has been found in our experiments (see Figs. 4, 5, 7, 8).

In view of our current knowledge, the acid shifts observed in spinal cord are likely to have multiple components, some of them could be related to depolarization-induced alkalinization of the glia. Acid shifts could be a result of: (1) Na^+-HCO_3^- cotransport into glial cells triggered by the rapid rise of $[K^+]_e$ during neuronal activity; (2) efflux of lactic acid from metabolically active neurones and glial cells; and (3) classic acid extrusion membrane transport systems in neurones and glial cells (Na^+/H^+ or $Na^+/HCO_3^-/Cl^-/H^+$), (for review see refs. 10, 57, 65). Similar to 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. Glial cells in tissue culture extrude lactic acid but only in response to the massive depolarization by large elevations of bath $[K^+]_e$ [65]. The superfusion of isolated frog spinal cord with NaF, which blocks the glycolytic portion of metabolic processes and production of lactate, had no effect on stimulation-evoked changes in pH_e[61]. Our present experiments revealed that activity-related acid shifts in pH_e mask the alkaline shifts resulting from neuronal activity. The acid shifts are closely related to gliogenesis and were absent in glial cell-depleted spinal cord PI. It is, therefore, reasonable to assume that glial cells regulate pH_e homeostasis at the expense of their intracellular pH.

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