GLIA 3:368–374 (1990)

Extracellular Potassium Influences DNA and Protein Syntheses and Glial Fibrillary Acidic Protein Expression in Cultured Glial Cells

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KEY WORDS Astrocytes

ABSTRACT Previous reports of increases in glial cell number and expression of glial fibrillary acidic protein (GFAP) in stimulated brain regions or epileptic tissue have implicated a role for increases in extracellular potassium concentration ($[K^+]_o$) in glial reactions. We examined the effects of altered $[K^+]_o$ on DNA and protein syntheses and GFAP expression of cultured glial cells isolated from the posthatch chick brain stem. $[K^+]_o$ was varied by adding both KCl and NaCl to K^+, NaCl-free medium to achieve final $[K^+]$ of 1–50 mM. DNA and protein syntheses were measured by incorporation of ^{3}H-thymidine and ^{3}H-leucine, respectively, into acid-insoluble material. GFAP expression was measured by a dot-immunoblotting assay. DNA synthesis in glial cells cultured in high (5–50 mM) K^+_o was 45–60% less than that of cells cultured in low (1–3 mM) K^+_o. Protein synthesis per cell was increased 34–44% in cells cultured in high K^+ as compared to those cultured in low K^+. GFAP expression was inversely related to $[K^+]_o$ over the 1–10 mM range. Compared to the baseline of 3 mM K^+_o, GFAP per cell was increased 65% at 1 mM and decreased 45% at 10 mM. These data suggest that increases in glial cell number and GFAP immunoreactivity found in sites of increased neuronal activity and in pathological tissues may not be caused solely by persistent increases in $[K^+]_o$. Instead, these results suggest that neuronal activity, through the release of K^+, may have an inhibitory influence on glial proliferation and GFAP expression. In light of work by others implying a relationship between GFAP immunoreactivity and rigidity of astroglial processes together with the data presented here, we suggest that the elevated $[K^+]_o$ accompanying neuronal activity, by inhibiting GFAP expression, may facilitate the morphological plasticity of glial cells. Conversely, conditions of low $[K^+]_o$ may contribute to rigidity of astrocytic processes.

INTRODUCTION

Extracellular potassium concentration ($[K^+]_o$) rises from a baseline of 3 mM to as much as 10 mM with neuronal activity (Heinemann and Lux, 1977; Somjen, 1979). In epileptic tissue, $[K^+]_o$ reaches 8–12 mM during ictal events (Moody et al., 1974; Somjen, 1979), and during anoxia and spreading depression, $[K^+]_o$s of 40–100 mM have been reported (Hertz, 1986; Sugaya et al., 1975).

$[K^+]_o$ is postulated to play an important role in neuron–glia interactions by signaling a need for increased metabolic support from surrounding glial cells during periods of increased neuronal activity (Pentreath, 1982; Pentreath and Kai-Kai, 1982). This hypothesis is based

Received December 18, 1989; accepted April 3, 1990.

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on two observations: a rise in $[K^+]_o$ accompanies neuronal activity (Heinemann and Lux, 1977; Moody et al., 1974), and a stimulation of glial cell metabolism occurs with increased $[K^+]_o$ (Pentreath, 1982). Specifically, elevated $[K^+]_o$ has been shown to stimulate oxygen consumption (Hertz, 1966; Hertz et al., 1973), glucose uptake and glycogen synthesis and turnover (Pentreath, 1982; Pentreath and Kai-Kai, 1982; Salem et al., 1975), protein synthesis (Lipton and Heimbach, 1977), and release of gamma-aminobutyric acid (Minchin and Iversen, 1974) in glial cells.

Gliosis, characterized by increases in glial cell proliferation and expression of glial fibrillary acidic protein (GFAP, an astrocytic intermediate filament), has been observed under conditions of altered neuronal activity or damage, such as around stab wounds (Adrian, 1968; (Bignami and Dahl, 1976; Cavanaugh, 1970; Latov et al., 1979) and adjacent to epileptic foci (Foerster and Penfield, 1930; Pollen and Trachtenberg, 1970; Ward, 1978). The frequent observations of gliosis adjacent to epileptic foci led Ward (1978) to propose that the high $[K^+]_o$ in epileptic tissue stimulates glial proliferation and contributes to scar formation.

Several investigators have reported changes in glial cell structure or number in response to presumed changes in the activity of local neurons (Altman and Das, 1964; Diamond et al., 1966; Gabbot et al., 1986; Hall and Borke, 1988; Kulenkampff, 1952; Salm et al., 1985; Watson, 1972). In all of these examples, however, the impact of the manipulation on neuronal activity was not measured. Further, the glial changes were observed after days or weeks.

In contrast, another type of glial response, following cessation of neuronal activity, has been observed in our laboratory (Canady and Rubel, 1989; Rubel and MacDonald, 1987). In this case, within 3 h of blocking all excitatory afferents to the chick cochlear nucleus, either by cochlea removal or by infiltration of tetrodotoxin into the perilymph, a dramatic increase in GFAP immunoreactive astrocytic processes was observed in the ipsilateral nucleus as compared to the control side of the same brain. It is unclear whether $[K^+]_o$ is altered in the previous examples of presumed activity change or in this example in which elimination of synaptic activity was verified. Because of the rapidity of the glial changes observed, we postulate that an activity-dependent signal, such as a change in $[K^+]_o$, is involved. Based on the above examples of glial changes in response to presumed increases in neuronal activity, one might favor Ward's hypothesis (1978) that increased $[K^+]_o$ stimulates glial reactions. Alternatively, the latter case of neuronal activity blockade suggests that conditions of low $[K^+]_o$ would stimulate glial reactions.

In the present study, we have investigated the effects of varied $[K^+]_o$ on the syntheses of DNA and protein and on the GFAP expression of cultured glial cells in order to determine whether responses of isolated glial cells mimic those seen in vivo following manipulations of neuronal activity.

MATERIALS AND METHODS

Cell Cultures

Glial cell cultures were established using a modification of the technique described by Ali-Osman et al. (1987) for establishing normal and neoplastic human glial cultures. Brain stems were removed from 4–5-day old hatchling chicks, minced, and incubated in an enzyme mixture (0.02% deoxyribonuclease, 0.04% collagenase, 0.2% neutral protease) at 37°C for 30–45 min. The cell suspension was then diluted with 5% fetal calf serum (FCS; Flow Laboratories, McLean, VA) in Dulbecco's modified Eagle's medium (DME; Gibco, Grand Island, NY) and filtered through a 60 μm mesh. Cells were collected by centrifugation at 300*g* for 12 min, resuspended in 30% FCS in DME, and plated in 75 cm^2 flasks at 4–5 $\times$ 10^4 cells per cm^2. The cultures were fed every 3–4 days with 20% FCS in DME.

These cell culture conditions were used to optimize the proportion of astrocytes. The nonastrocytic (GFAP-negative) population was estimated to be less than 5%, including an oligodendrocyte (galactocerebroside-positive) population of less than 3% of all cells (unpublished observations). Neurons would not be expected to survive under these conditions due to the maturity of the tissue of origin (Sensenbrenner, 1977), and no cells morphologically resembling neurons were observed.

When the monolayer cultures reached confluence (after 8–12 days of incubation), the cells were harvested using 0.25% trypsin (Sigma, St. Louis, MO) and replated, using the experimental media described below, in either 96-well microtiter plates or 12-well culture plates. Low and high density cell cultures were obtained by seeding 1.25 $\times$ 10^4 or 2.5 $\times$ 10^4 cells per microtiter plate well. Cells were seeded in the 12-well plates at 1.5-2 $\times$ 10^5 cells per well.

Experimental Media

K^+_o was varied at constant osmolarity by supplementing DME from which both KCl and NaCl had been omitted (Gibco, Grand Island, NY) with KCl and NaCl such that the combined [KCl] and [NaCl] was the same as in normal DME, 115 mM. No FCS was added to the media for short-term experiments (24 h), but for long-term experiments (4 days), media were supplemented with 0.5% FCS to sustain cell viability. Final $[K^+]$s were 1, 3, 5, 10, and 50 mM. All of the experimental media were sterilized by passage through a 0.22 μm filter.

DNA and Protein Syntheses Assays

DNA and protein syntheses were measured as the cellular incorporation of ^{3}H-thymidine and ^{3}H-leucine, respectively, into acid-insoluble material. The techniques used were a modification of those described by Ali-Osman and Maurer (1980). Twenty-four hours after seeding cells in the 96-well microtiter plates, the cul-

tures were re-fed with 200 μl of the experimental media described above plus either 1 μCi of ^{3}H-thymidine (NEN Research Products, Boston, MA; specific activity: 20 Ci/mmol) or 2 μCi of ^{3}H-leucine (NEN Research Products; specific activity: 60 Ci/mmol) per well. The cells were trypsinized 24 h later and collected onto glass fiber filter paper using a cell harvester (Cambridge Technologies, Cambridge, MA). The samples were washed with distilled water and 5% trichloroacetic acid, and the precipitated DNA or protein was transferred to glass vials and prepared for liquid scintillation counting using Biofluor scintillation fluid (NEN Research Products). The duration of the pre-incubation and radionuclide pulse times (24 h each) were chosen after optimization studies indicated that relative amounts of ^{3}H-thymidine incorporation as a function of $[K^+]_o$ are consistent across pre-incubation periods of 3–48 h and ^{3}H-thymidine pulses of 5–48 h.

To determine the effects of varied $[K^+]_o$ on cell number, cells plated in 12-well plates were pre-incubated for 24 h followed by a 24 h exposure to the experimental media. These cells were removed from the plates with trypsin and counted on a hemacytometer.

GFAP Immunoblotting Assay

Cells were cultured in 12-well plates for 4 days in experimental media plus 0.5% FCS. Duplicate cultures were set up: one culture of a pair was trypsinized and the cells counted on a hemacytometer; the other was scraped up with a rubber policeman and assayed for protein and GFAP content. For the latter, cells were collected in 1 ml sample buffer (0.4 M NaCl, 10 mM HEPES, 1% SDS; pH 7.0), homogenized and pelleted for 3 min at 8,160*g*. Protein assays were done according to the method of Schaffner and Weissman (1973) using an Amido Schwartz stain, and the dot-blot method was a modification of that of Carlson and Kelly (1983). Cell extracts were spotted onto nitrocellulose filters, incubated overnight in 5% bovine serum albumin in Tris buffered saline (0.9% NaCl, pH 7.4) at 4°C, and then immersed in a 1:750 dilution of GFAP antiserum for 2 h. Following a series of washes, the filters were placed in ^{125}I-labeled goat anti-rabbit serum (NEN Research Products, Boston, MA; specific activity: 8.2 μCi/mg) for 45 min, washed in 3 changes of Tris buffered saline, dried overnight, and exposed to autoradiographic film at −70°C for 2–4 days. The density of the resulting dots on film was analyzed with a BioQuant microdensitometry system (R&M Biometrics, Nashville, TN).

RESULTS

Effects of K^+ on DNA Synthesis

As shown in Fig. 1, at all $[K^+]_o$s above the 3 mM baseline, DNA synthesis was suppressed 45–60% (± 4–6%, SEM, six experiments). At 1 mM K^+_o, DNA synthesis was only 8% (± 5%) less than baseline. The cell count data (Fig. 2; four experiments) are similar with the exception of 5 mM K^+_o. At 5 mM K^+_o, ^{3}H-thymidine incorporation was reduced 49% (± 4%) from the 3 mM baseline, while cell number was increased 5% (± 1.5%). At 1 mM K^+_o, cell number was within 1 standard error of baseline. At 10 mM K^+_o, cell number was 20% (± 15%) less than baseline, and at 50 mM K^+_o, cell number was 40% (± 11%) less than baseline. Thus, DNA synthesis per cell (Fig. 3) is not constant across

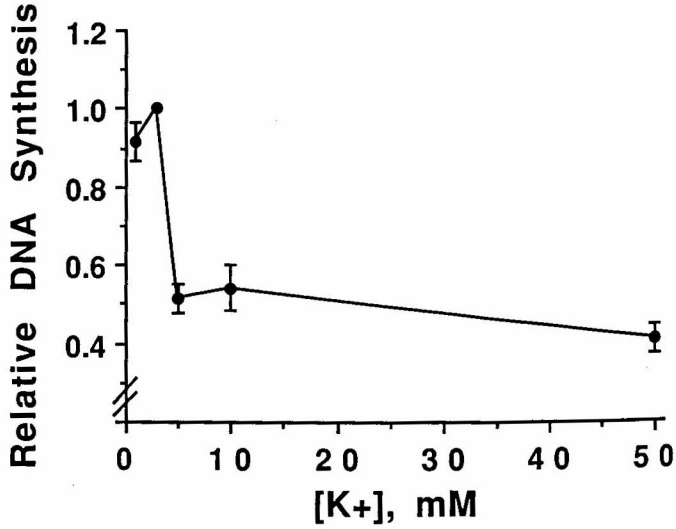

Fig. 1. Effects of $[K^+]_o$ on DNA synthesis. Mean ^{3}H-thymidine incorporation (in cpm) is expressed as a ratio to mean incorporation by cells cultured in 3 mM KCl. KCl and NaCl were added to KCl-, NaCl-free DME such that the combined [KCl] and [NaCl] was kept constant at 115 mM for [KCl] of 1, 3, 5, 10, 50 mM. Ratios averaged across six experiments. Bars in this and following figures represent standard error of the mean (SEM) for ratios averaged across experiments.

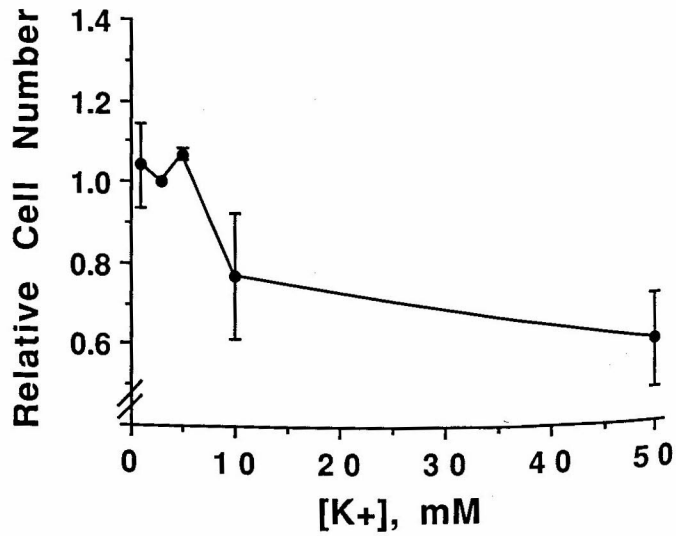

Fig. 2. Effects of $[K^+]_o$ on cell number. Mean cell number per experimental condition is expressed as a ratio to mean cell number for cultures exposed to 3 mM KCl for 24 h. Ratios averaged across four experiments. The error bar for 5 mM KCl is slightly larger than the size of the symbol.

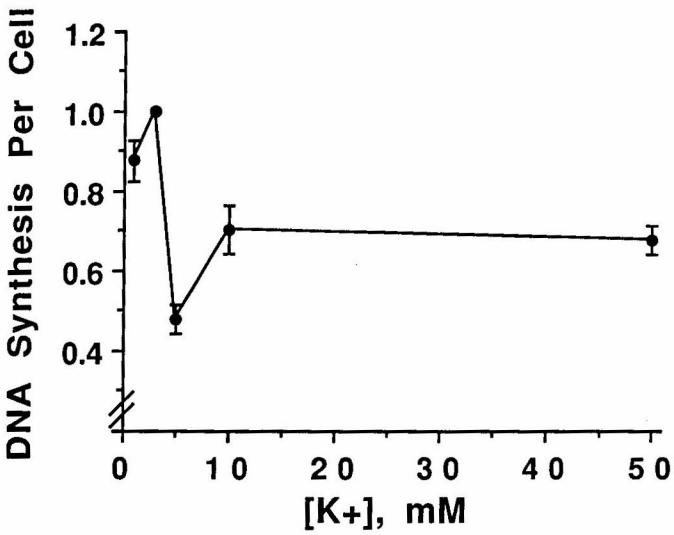

Fig. 3. Effects of $[K^+]_o$ on DNA synthesis per cell. Ratios representing relative ^{3}H-thymidine incorporation (from Fig. 1) were divided by ratios representing relative cell number (from Fig. 2). Error bars represent SEM of the numerator (^{3}H-thymidine incorporation) only.

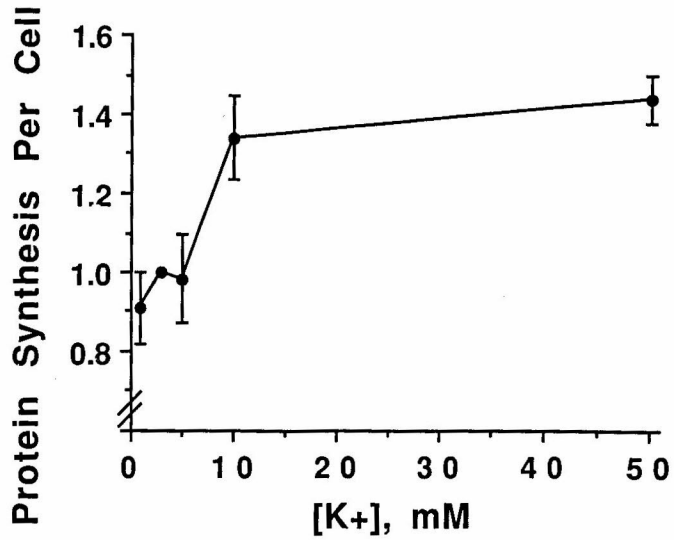

Fig. 4. Effects of $[K^+]_o$ on protein synthesis per cell. Ratios representing relative ^{3}H-leucine incorporation were averaged across six experiments. These ratios were then divided by ratios representing relative cell number. Error bars represent SEM of the numerator (^{3}H-leucine incorporation) only.

varied $[K^+]_o$s. DNA synthesis on a per-cell basis dropped dramatically at 5 mM versus 3 mM K^+_o and was less reduced at higher $[K^+]_o$s.

Neither DNA synthesis nor protein synthesis as a function of $[K^+]_o$ was influenced by cell plating density. Therefore, data were combined across the high and low plating densities.

Effects of K^+ on Protein Synthesis

Fig. 4 (six experiments) shows that protein synthesis, expressed on a per-cell basis, increased by 34% (± 10%) at 10 mM and by 44% (± 6%) at 50 mM K^+_o as compared to the 3 mM baseline condition. Little to no effect of 1 and 5 mM K^+_o on protein synthesis was observed.

Effects of K^+ on GFAP Expression

An inverse relationship between $[K^+]_o$ and cellular GFAP content was observed (Fig. 5; four experiments). There was a 65% (± 12%) increase in GFAP in cells grown in 1 mM versus 3 mM K^+_o. GFAP per cell at 5 and 10 mM K^+_o was decreased 22% (± 18%) and 45% (± 26%), respectively, relative to GFAP per cell at 3 mM. A small decrease (13% ± 11%) in GFAP per cell was observed at 50 mM K^+_o as compared to 3 mM. For all dot-blots, variations in GFAP content were paralleled by variations in protein content, as determined by the Amido Schwartz assay.

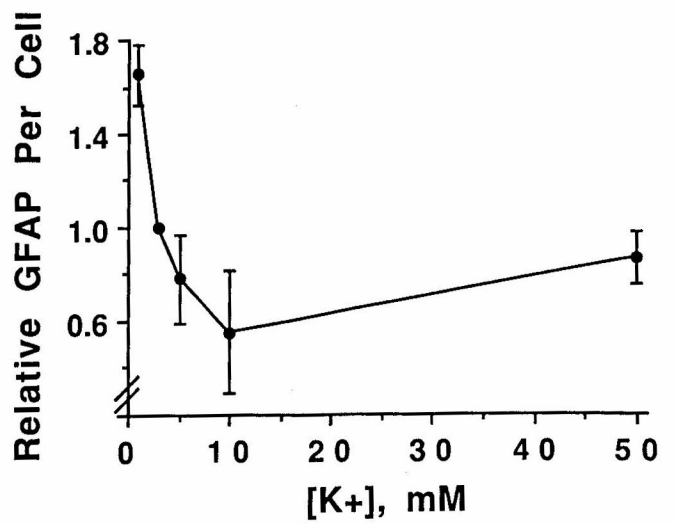

Fig. 5. Effects of $[K^+]_o$ on GFAP expression. Ratios based on autoradiographs of dot-immunoblots. Density of sample dots was compared to a standard curve derived from a partially purified GFAP preparation. Ratios thus represent μgs GFAP per cell in samples exposed to experimental media divided by μgs GFAP per cell in samples exposed to 3 mM KCl. Ratios were averaged across four experiments.

DISCUSSION

We have shown that DNA synthesis and GFAP expression in cultured astrocytes are maximal at low (1–3 mM) $[K^+]_o$ and reduced at higher (5–50 mM) concentrations, while protein synthesis is increased at 10–50 mM K^+_o. These results suggest that increased glial cell mass and GFAP expression may occur in regions of decreased neuronal activity and low $[K^+]_o$. These results also suggest that neuronal activity, through the release of K^+, may inhibit glial cell proliferation and GFAP expression. Further, it would appear that previously observed increases in glial cell mass and GFAP expression in areas of presumed increases in neuronal activity may

not be a direct result of presumed concurrent increases in $[K^+]_o$.

Effects of K^+ on DNA and Protein Syntheses

The reduction in DNA synthesis at $[K^+]_o$ greater than 3 mM suggests that high $[K^+]_o$ inhibits glial cell proliferation in culture. The large decrease in DNA synthesis observed when $[K^+]_o$ was raised from 3 to 5 mM was not accompanied by a decrease in cell number. This disparity between DNA synthesis and cell number (Fig. 3) suggests that fewer cells progress to S-phase at 5 mM K^+_o, yet these cells remain viable during the 24 h period. However, at higher $[K^+]_o$, both DNA synthesis and cell number are reduced, suggesting more cell loss under these conditions in addition to a suppression of cell proliferation.

As shown in Fig. 4, glial cells synthesize more protein when cultured in high (10–50 mM) $[K^+]_o$. This suggests that dying cells, or non-proliferative cells which survive in the face of challenge to their ionic environment, increase their protein synthesis. The increase in overall protein synthesis measured after 24 h, however, is not reflected in increased GFAP nor in total protein content after 4 days. Either the newly synthesized proteins are degraded or protein synthesis decreases between 1 and 4 days of exposure to high $[K^+]_o$. Since altering $[K^+]_o$ affects glial cell proliferation, we report protein synthesis on a per-cell basis, rather than per cell population. The decrease in protein synthesis of glial cell populations exposed to 105 mM K^+_o reported by Takahashi et al. (1970) actually may have been an increase in protein synthesis if analyzed on a per-cell basis.

Effects of K^+ on GFAP Expression

GFAP expression was highest in glial cells cultured in 1 mM K^+_o, the lowest $[K^+]_o$ studied. A similarly low $[K^+]_o$ occurs transiently in vivo before restoration of the normal 3 mM baseline after $[K^+]_o$ rises with neuronal activity or spreading depression. Other conditions in which K^+_o would be decreased are not known at present. A persistent condition of 3 mM K^+_o, as in our glial cell cultures, may mimic an effect of preventing action potentials in an otherwise active neuronal population. Thus a lack of repeated activity-related increases in $[K^+]_o$ might stimulate the observed increase in GFAP immunoreactivity in the silenced cochlear nucleus (Canady and Rubel, 1989; Rubel and MacDonald, 1987).

The decreased GFAP expression at higher K^+_os (within the physiological range, 3–10 mM) may be an indication that astrocytes are morphologically more adaptive when cultured in these conditions. This is supported by the observation of Duffy et al. (1982) that cells and processes lightly and diffusely GFAP-positive showed the most morphologic changes, while intensely stained processes appeared quite rigid and were capable of only simple extension–retraction movements. Light, diffuse staining for GFAP was also found in the redistributed astrocytes of the supraoptic nuclei in lactating rats (Salm et al., 1985), another suggestion that more plastic astrocytes express less GFAP. Thus by inhibiting GFAP, activity-related increases in $[K^+]_o$ may facilitate morphological changes in glial cells while also stimulating the synthesis of proteins other than GFAP.

One might expect GFAP expression to increase at the very high $[K^+]_o$ of 50 mM since this would be associated with pathological conditions such as anoxia and spreading depression (Hertz 1986; Sugaya et al., 1975). Instead, we found GFAP per cell at 50 mM K^+_o to be 13% lower than in cells cultured in our baseline (3 mM K^+_o) condition. Thus large increases in $[K^+]_o$ may not contribute to increased GFAP expression under these pathological conditions.

Comparison to In Vivo Studies

Three factors should be considered when relating the present in vitro data to previous in vivo studies. First, the culture medium creates a very large extracellular space in which K^+ is chronically altered. In vivo, $[K^+]_o$ would return to baseline after fluctuations within the physiological range, and those fluctuations in $[K^+]_o$ would likely be pulsatile rather than sustained. Second, astrocytes in situ are in a different proliferative state than those in vitro. Hatten (1985) observed that cultured astrocytes stop proliferating in the presence of neurons. While it would be interesting to know the effects of varied $[K^+]_o$ on the proliferation of astrocytes cultured in the presence of neurons, one would have difficulty distinguishing between the direct effects of K^+ and the effects of any other signals which might be released by neurons which have been depolarized by increased $[K^+]_o$. One might get around this confound of the effects of K^+-depolarized neurons by culturing the astrocytes in the presence of a neuronal membrane preparation which also inhibits glial proliferation (Hatten, 1987). Finally, immature glial cells, such as those used in cell culture, may respond to damage or to changes in $[K^+]_o$ differently than would mature astrocytes. Bignami and Dahl (1976) found that the astroglial response to stab wounds, as measured by immunostaining for GFAP, was markedly attenuated in neonatal rats versus adults.

The in vivo evidence for increases in glial cell number, mass or GFAP expression in conditions of high $[K^+]_o$ comes from studies of two types of tissue: epileptic foci (Foerster and Penfield, 1930; Pollen and Trachtenberg, 1970; Ward, 1978) and stimulated (directly or indirectly) brain nuclei (Hall and Borke, 1988; Kulenkampff, 1952; Watson, 1972). Glial changes in epileptic foci and stimulated neural tissue may occur in the presence of active neurons and increased $[K^+]_o$. If so, unknown factors may negate and actually reverse the changes expected when only $[K^+]_o$ is manipulated.

Alternatively, the epileptic and stimulated tissue in which glial reactions have been described could also be

sites of excitotoxic neuronal degeneration (Coyle, 1987; Rothman, 1984). In such a case, the glial changes might occur in sites of neuronal death (or eventual death), where activity has ceased and $[K^+]_o$ may not be elevated. This hypothesis of glial reactions to decreased neuronal activity would be consistent with our previous observations (Canady and Rubel, 1989; Rubel and MacDonald, 1987) of increased GFAP in the silenced cochlear nucleus, where activity ceases and a portion of the neurons eventually die.

We suggest that neuronal activity, through the release of K^+ into the extracellular space, may regulate the GFAP expression and morphology of astrocytic processes. Active neurons would maintain GFAP expression at a normal, minimal level, facilitating the morphological plasticity of astrocytic processes. Inactive or dying neurons would fail to release K^+ into the extracellular space and thereby allow increased GFAP expression and possibly increased glial process extension and rigidity.

It is likely that altered $[K^+]_o$ exerts its effects on glial proliferation and GFAP expression indirectly, as changes in $[K^+]_o$ are known to stimulate a variety of cellular events. For example, elevated $[K^+]_o$ stimulates Ca^{++} uptake (Hertz et al., 1989), depolarizes glial membranes (Sugaya et al., 1979), lowers intracellular $[Na^+]$, and increases intracellular $[Cl^-]$ (Walz and Hertz, 1983), activates the Na^+-K^+ ATPase (Walz and Hertz, 1982) and, at very high concentrations, causes astrocytic swelling (Walz and Hertz, 1983; Walz and Mukerji, 1988). Some of these potential mechanisms could be tested by repeating the present experiments with Ca^{++}-free or Cl^--free media.

ACKNOWLEDGMENTS

We thank Dr. Dianne Durham and Judith Debel for advice and assistance on the immunoblotting studies, and Nell Cant, Dianne Durham, Stephen Gammie, P. Vijay Sarthy, and Phillip Schwartzkroin for their helpful comments on earlier versions of the manuscript. We also thank Dr. Sarthy for generously providing the partially purified GFAP preparations. This work was supported by PHS grants DC00393 and GM07108. Preliminary reports of these data have appeared in abstract form (Canady et al., 1987, 1988).

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