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Acidic pH Rapidly Increases Immunoreactivity of Glial Fibrillary Acidic Protein in Cultured Astrocytes

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ABSTRACT Neuroepithelial progenitor cells from forebrains of newborn rat pups develop into "mature" astrocytes in an epidermal growth factor-containing medium free of serum (Von Visger et al: Exp Neurol 128:34, 1994). Eight-week-old "mature" astrocyte cultures on poly-L-lysine-coated dishes were exposed to an acidic medium (pH 5.8–6.0) for 2–6 h. Immunoreactivity for glial fibrillary acidic protein (GFAP) dramatically and rapidly increased; this immediate increase was not affected by pretreatment with cycloheximide. In further experiments we found that the increase in GFAP was undiminished for 24–48 h after the acid-treated astrocytes were returned to normal growth medium. The Ca^{2+} channel antagonists nifedipine and diltiazem *attenuated* the increase in GFAP immunoreactivity. These results suggest that extracellular acidosis may produce a rapid increase in GFAP immunoreactivity in astrocytes independent of de novo protein synthesis, possibly by increasing intracellular levels of free Ca^{2+} ions.

INTRODUCTION

Reactive gliosis is characterized by markedly increased immunocytochemical staining for glial fibrillary acidic protein (GFAP; Eng. 1988). Reactive gliosis in the central nervous system (CNS) is induced by a variety of insults, including seizure. The earliest time reported for a seizure-induced increase in GFAP gene expression is 24 h after the seizure (Steward et al., 1991). Our recent findings indicate that a seizure-inducing dose of the anticholinesterase soman produces a marked increase in GFAP immunoreactivity in piriform cortex within 1 h of the seizure (Shipley et al., 1990). This finding indicates that activation of astrocytes in response to seizure occurs much earlier than previously reported.

Little is known about the cellular and molecular signals that initiate the astroglial response to seizures. Seizures cause a dramatic acceleration in oxygen consumption and an increase in glycolytic flux (Wasterlain, 1989). This marked increase in anaerobic glycolysis is accompanied by a large increase in lactic acid levels and a dramatic fall in the extracellular pH (Flynn et al., 1989; Meric et al., 1994; Nemoto, 1985; Wasterlain, 1989). Therefore, we hypothesized that decreased pH might be one of the signals for reactive gliosis.

We recently described a new cell culture system for astrocytes utilizing multipotential progenitor cells from newborn rat forebrains (Von Visger et al., 1994; Reynolds and Weiss, 1992). The astrocytes that develop in this culture undergo a developmental increase in GFAP and a concomitant decrease in vimentin, events that mirror astroglial maturation seen in vivo (Bignami et al., 1982; Dahl, 1981). Furthermore, unlike cultures grown in serum, in which both GFAP and vimentin levels remain very high, astrocytes in these cultures eventually express only moderate steady state levels of GFAP with little or no expression of vimentin. This situation closely mimics the situation in vivo making

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this culture system suitable for investigations of potential signals for reactive gliosis.

Here we show that decreasing the pH of such cultures to 5.8–6.0 provokes a rapid increase in immunoreactive GFAP. This increase occurs independently of de novo protein synthesis. Furthermore, the increase in GFAP is diminished by nifedipine or diltiazem, Ca^{2+} channel blockers.

MATERIALS AND METHODS

Cell Culture

Primary progenitor-cell cultures were prepared from the forebrains of 1–2-day-old Fisher rats as described previously (Von Visger et al., 1994). Cells were resuspended in serum-free medium (SFM) at 5×10^5 cells/ml and maintained in SFM at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 4–6 days (Von Visger et al., 1994). Floating cell clusters were collected and replated (secondary cultures) on 6-well culture plates (Linbro) coated with poly-L-lysine (Sigma). These cultures were grown in a *modified*-serum-free medium (*without* Ham's F-12) consisting of DMEM (6.0 g glucose/L; Gibco) containing 50 U/ml penicillin/streptomycin (Gibco), plus 50 nM hydrocortisone, 100 μM putrescine, 30 nM selenium, 20 μg/ml transferrin, 10 μg/ml insulin, 500 ng/ml prostaglandin $F_{2\alpha}$, 20 ng/ml mouse salivary gland epidermal growth factor (EGF), and 5 mM HEPES, pH 7.2 (all from Sigma, St. Louis, MO). Half the volume of medium was exchanged every 7 days with fresh modified SFM. These cultures were characterized in a recent full-length report (Von Visger et al., 1994).

Experimental Treatment

Eight-week-old astrocyte cultures derived from neuroepithelial progenitor cells were titrated with either 0.1 N HCl or with 0.25 M lactic acid (Sigma)/0.25 N HCl until the pH reached 5.8–6.0; the latter treatment was designed to mimic lactic acidosis. The treated cultures were maintained in a 37°C incubator for 2–6 h. Some cultures were treated, washed, and maintained in fresh modified SFM for 24–48 h. In other experiments, cultures were pretreated for 30 min with cycloheximide (15 μg/ml, Sigma), diltiazem (100 μM, Sigma), or nifedipine (100 μM; RPI). Each experiment was repeated three or more times.

Immunocytochemistry

Cultures were fixed and processed for immunocytochemistry as described previously (Oh et al., 1993; Zimmer et al., 1995). Polyclonal rabbit antibody against bovine GFAP (Dako, Inc.) was used at a dilution of 1:25,000. The primary antibody was visualized using the horseradish peroxidase (HRP)-immunopure ABC method (Pierce Chemical Co).

RESULTS

In order to investigate signals that might mediate reactive astrogliosis after the onset of seizures, we initially tested a number of experimental perturbations to determine their effects on GFAP immunoreactivity. Seizures in vivo are associated with intense neural activity involving glutamatergic synapses, increased extracellular K^+, and increased lactate production (Chapman et al., 1984; Chastain et al., 1989; Meric et al., 1994). By contrast, interleukin-1β may mediate reactive astrogliosis in stab injuries (Giulian and Lachman, 1985). Any of these changes could function as a signal mediating reactive gliosis in seizures.

Cultures treated for 2–24 h with L-glutamate (up to 100 μM), K^+ ions (up to 150 mM), and interleukin-1β (up to 1 μg/ml) showed no change in GFAP (not shown). However, reducing the pH of the medium for 1–2 h to 5.8–6.0 either with 0.1 N HCl alone (not shown) or with a combination of 0.25 N lactic acid/0.25 N HCl to mimic acidosis caused a dramatic increase in GFAP immunoreactivity (Fig. 1C,D). Trypan blue exclusion tests indicated that astrocytes survived well in low pH conditions for up to 6 h in culture. Furthermore, in low pH medium, many astrocytes with a flat, polygonal morphology rapidly (within 15–30 min) transformed into stellate-shaped cells in culture (Fig. 1A,B). Pretreating cultures for 30 min with cycloheximide had no effect on any of these changes, suggesting that the responses did not require de novo synthesis of the protein (not shown).

In order to determine if Ca^{2+} influx was involved in the increase in GFAP, we pretreated some cultures with the Ca^{2+} channel blockers nifedipine or dilitazem (100 μM) before lowering pH. As shown in Figure 1F, nifedipine mitigated the effect of pH on GFAP. As both of these drugs block Ca^{2+} ion influx, it could be that the overall effect of lowering pH is mediated, at least in part, by the sudden influx of Ca^{2+} ions into the cytosol. Further studies are needed to test this hypothesis.

Experiments with cycloheximide indicated that the rapid initial increase in GFAP immunoreactivity resulting from a 2 h lowering of pH did not require protein synthesis. We wished to know, however, whether the increase in GFAP immunoreactivity, regardless of its cause, was transient or would persist. We lowered the pH of cultures for 2–6 h, washed the cultures, added fresh modified SFM, and incubated the cultures for 24–48 h. As illustrated in Figure 1E, it is clear that the increase in GFAP levels produced by acidic pH persists for at least 24–48 h in culture.

DISCUSSION

Little is known about the signals that induce reactive gliosis. Seizures in the rat produce a significant increase in lactate and an acidification of extracellular fluid within minutes after the onset of seizures (Meric et al., 1994; Wasterlain, 1989). The present findings thus suggest that an acidic extracellular pH, whether

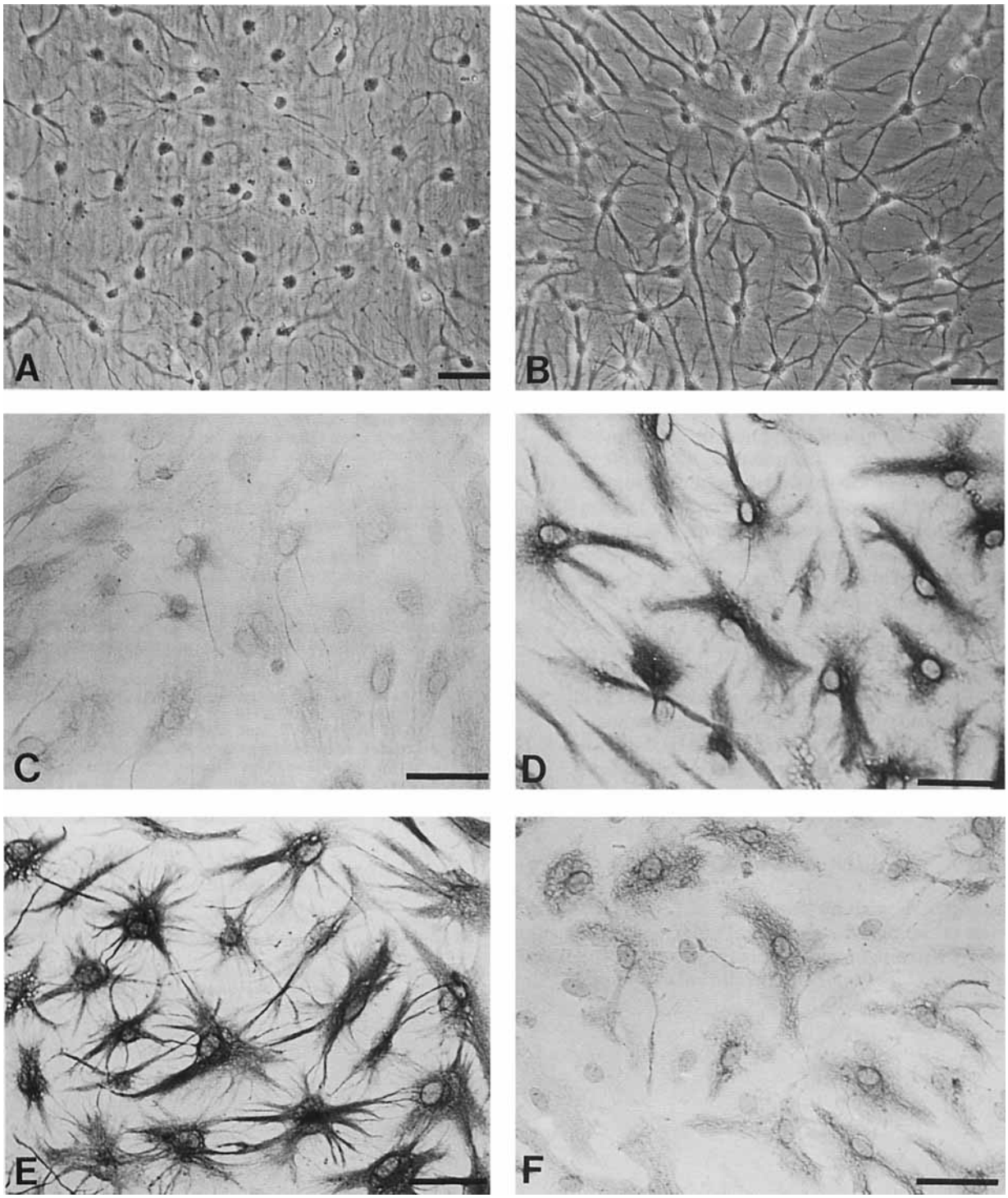

Fig. 1. Effect of acidic pH on the morphology and GFAP immunoreactivity of cultured astrocytes. Eight-week-old cultures were exposed to an acidic medium (pH 5.8) for 2 h and processed for GFAP immunocytochemistry. Phase-contrast micrographs of living untreated, control cultures at pH 7.2 (**A**) and cultures exposed to an acidic medium for 30 min (**B**). GFAP immunoreactivity of untreated control cultures (**C**), cultures treated with low pH for 2 h (**D**), cultures treated with low pH for 2 h followed by washing and incubation with fresh *modified* SFM for 48 h (**E**), and cultures treated with a Ca^{2+} channel blocker, nifidepine (100 μM), for 30 min and then with low pH for 2 h (**F**). Bar = 50 μm.

mediated by an increase in $[H^+]$ ions alone or by an increase in both $[H^+]$ and [lactate], could be part of the signalling pathway that leads to the rapid rise in GFAP immunoreactivity following seizures. It should be pointed out that increasing [lactate] alone did not produce an increase in GFAP immunoreactivity unless the pH was

concomitantly lowered with HCl (not shown). As the increased GFAP immunoreactivity in cultured astrocytes was not blocked by cycloheximide, it is possible that an acidic pH induces an increase in antigenic epitopes. This could involve a posttranslational modification such as phosphorylation. Alternatively, there could be an increased accessibility of GFAP antibodies to the internal domain of the filaments (Eng et al., 1989), associated with changes in astrocyte morphology. This might occur, for example, if astrocytes attempt to increase their surface area to buffer increased extracellular ions or to increase their contact with hyperactive neurons. Consistent with the latter possibility is the finding that cultured astrocytes showed rapid morphological changes in response to H^+/lactate.

We found an increase in GFAP immunoreactivity in astrocytes within 1 h of soman-induced seizures in vivo (Shipley et al., 1990). This increase was preceded by increased *Fos* in neighboring neurons within 30 min of the onset of seizures (Zimmer et al., 1995). In situ, hyperactive neurons would respond to their increased rate of firing by producing lactate. This lactate might then diffuse from the neurons, lowering interstitial pH. Astrocytes, in turn, would respond to the decrease in pH and the influx of Ca^{2+} by changing their surface area to buffer the extracellular ionic changes. An early response to these changes might be a rapid change in astrocytic morphology followed by a transient increase in GFAP immunoreactivity independent of protein synthesis. Should the extracellular pH changes persist, the increase would ultimately result in the synthesis of new mRNA for GFAP. This protein would be detectable in a second, more refractive phase that would appear hours later and persist for several weeks. Future experiments will test this hypothetical chain of events.

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