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Epidermal Growth Factor Receptor Agonists Increase Expression of Glutamate Transporter GLT-1 in Astrocytes through Pathways Dependent on Phosphatidylinositol 3-Kinase and Transcription Factor NF-κB

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ABSTRACT

The glial glutamate transporter GLT-1 may be the predominant Na^+-dependent glutamate transporter in forebrain. Expression of GLT-1 correlates with astrocyte maturation in vivo and increases during synaptogenesis. In astrocyte cultures, GLT-1 expression parallels differentiation induced by cAMP analogs or by coculturing with neurons. Molecule(s) secreted by neuronal cultures contribute to this induction of GLT-1, but little is known about the signaling pathways mediating this regulation. In the present study, we determined whether growth factors previously implicated in astrocyte differentiation regulate GLT-1 expression. Of the six growth factors tested, two [epidermal growth factor (EGF) and transforming growth factor-α] induced expression of GLT-1 protein in cultured astrocytes. Induction of GLT-1 protein was accompanied by an increase in mRNA and in the V_{max} for Na^+-dependent glutamate transport activity. The effects of dibutyryl-cAMP and EGF were additive but were independently blocked by inhibitors of protein kinase A or protein tyrosine kinases, respectively. The induction of GLT-1 in both EGF- and dibutyryl-cAMP-treated astrocytes was blocked by inhibitors targeting phosphatidylinositol 3-kinase (PI3K) or the nuclear transcription factor-κB. Furthermore, transient transfection of astrocyte cultures with a constitutively active PI3K construct was sufficient to induce expression of GLT-1. These data suggest that independent but converging pathways mediate expression of GLT-1. Although an EGF receptor-specific antagonist did not block the effects of neuron-conditioned medium, the induction of GLT-1 by neuron-conditioned medium was completely abolished by inhibition of PI3K or nuclear factor-κB. EGF also increased expression of GLT-1 in spinal cord organotypic cultures. Together, these data suggest that activation of specific signaling pathways with EGF-like molecules may provide a novel approach for limiting excitotoxic brain injury.

The acidic amino acid glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (CNS). Low extracellular concentrations of glutamate, a prerequisite for the high signal-to-noise ratio of synaptic communication, are maintained by a family of Na^+-dependent transporters (for review, see Sims and Robinson, 1999). These proteins, which rapidly clear glutamate from the synaptic cleft, are essential for signal termination, neurotransmitter recycling, and prevention of excitotoxicity. Five high-affinity subtypes, identified by molecular cloning, are differentially expressed throughout the CNS. EAAC1 (EAAT3) and EAAT4 are localized predominantly in neurons, and EAAT5 is enriched in retinal tissue, whereas GLAST (EAAT1) and GLT-1 (EAAT2) are generally expressed in astrocytes (for review, see Sims and Robinson, 1999).

Both in vivo and in vitro studies have provided compelling evidence that transport into astrocytes is the predominant route for clearance of extracellular glutamate and for limiting excitotoxicity. Several reports indicate that in neuronal culture models, Na^+-dependent transport into astrocytes attenuates glutamate toxicity (for review, see Robinson and

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ABBREVIATIONS: CNS, central nervous system; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor-α; dbcAMP, dibutyryl-cAMP; NCM, neuron-conditioned medium; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; PDTC, pyrrolidinedithiocarbamate; ECL, enhanced chemiluminescence; NGF, nerve growth factor; PDGF, platelet-derived growth factor; Bis II, bisindolylmaleimide II; bFGF, basic fibroblast growth factor; TBS, Tris-buffered saline; TGT, TBS containing 5% normal goat serum and 0.1% Triton X-100; GFP, green fluorescent protein; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PLCγ, phospholipase Cγ; MAP, mitogen-activated protein; MEK, MAP kinase kinase; Erk, extracellular signal receptor-activated kinase; PKC, protein kinase C; NF-κB, nuclear factor-κB.

Dowd, 1997). Furthermore, gene deletion, gene knockdown, and pharmacological studies indicate that the GLT-1 subtype may contribute up to 90% of total transport in the forebrain (for review, see Robinson, 1999). Transport deficiency and down-regulation of GLT-1 and/or GLAST are associated with neurodegenerative disorders such as amyotrophic lateral sclerosis, epilepsy, hypoxia/ischemia, and head trauma (for review, see Sims and Robinson, 1999). Therefore, up-regulation of glial transporters may be a promising strategy for the treatment and/or prevention of neurodegeneration accompanying CNS insults. GLT-1, a major glial transporter, would be an appropriate target for such a strategy.

Although the genes have been identified for these transporters, the promoter elements have not been characterized, and the mechanisms regulating their expression remain unclear. There is evidence to suggest that induction of GLT-1 is associated with astrocyte differentiation, but very little is known about the mechanisms involved (Swanson et al., 1997; Schlag et al., 1998). In vivo, the expression of GLT-1 changes dramatically during development with low levels in the early postnatal period and a rapid increase during synaptogenesis (for review, see Sims and Robinson, 1999). In contrast to mature astrocytes in vivo, primary astrocytes in culture express essentially no GLT-1 protein and thus can be used as a model system to identify the molecular mechanisms controlling transporter expression. cAMP analogs and coculturing with neurons stimulate expression of GLT-1 in these astrocytes (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al., 1998). In both cases, induction of GLT-1 is associated with differentiation of astrocytes. The effects of coculturing with neurons can be at least partially attributed to the release of a secreted molecule. Although both neurons and astrocytes in cocultures release various types of molecules, epidermal growth factor receptor (EGFR) agonists have been strongly implicated in the regulation of proliferation and differentiation of astrocytes in vitro and in vivo (see *Discussion*). In the present study, we demonstrate that growth factors that act through EGFR [EGF and transforming growth factor-α (TGFα)] induce expression of GLT-1 in primary astrocytes in culture. The effects of cell permeable inhibitors of various signaling pathways activated by either dibutyryl-cAMP (dbcAMP) or EGF were examined. Although some of these inhibitors selectively blocked the effects of either dbcAMP or EGF, others blocked the effects of both EGF and dbcAMP. Similarly, some of the same inhibitors blocked the neuron-conditioned medium (NCM)-mediated induction of GLT-1. These studies suggest that dbcAMP, EGF, and NCM induce GLT-1 expression through activation of the same signaling pathways.

Experimental Procedures

Materials. Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT); all other cell culture reagents were from Gibco-BRL (Gaithersberg, MD). Anti-glial fibrillary acidic protein (GFAP) antibody, poly-D-lysine, antiactin antibody, dbcAMP, pyrrolidinedithiocarbamate (PDTC), and porcine pancreas insulin were obtained from Sigma Chemical Co. (St. Louis, MO). L-[^{3}H]glutamate and [α-^{32}P]deoxycytidine 5′-triphosphate were obtained from DuPont/NEN (Boston, MA). Donkey anti-rabbit horseradish peroxidase IgG, rainbow molecular weight markers, Hybond N+, and enhanced chemiluminescence (ECL) kits were purchased from Amersham (Arlington Heights, IL). Immobilon P membrane was from Millipore (Bedford, MA). Dihydrokainate was purchased from Genosys (The Woodlands, TX). 7S Nerve growth factor (mouse submaxillary gland) (NGF), human recombinant platelet-derived growth factor (BB homodimer) (PDGF), genistein, PD98059, tyrphostin A25, wortmannin, bisindolylmaleimide II (Bis II), and KT5720 were purchased from Calbiochem (La Jolla, CA). Mouse recombinant EGF, basic fibroblast growth factor (bFGF) and TGF-α were obtained from Collaborative Biomedical Products (Bedford, MA). LY294002 was obtained from Biomol (Plymouth Meeting, PA). All growth factors and PDTC were dissolved in sterile deionized water. All inhibitors were dissolved in dimethyl sulfoxide. GenePorter transfection reagent was purchased from Gene Therapy Systems (San Diego, CA). Immumount was purchased from Shandon (Pittsburgh, PA). Anti-mouse IgG and IgM-fluorescein and anti-rabbit IgG-rhodamine conjugates were obtained from Jackson ImmunoResearch (West Grove, PA). A2B5 antibody was made as previously described (for original citation, see Grinspan et al., 1996). Rabbit complement was purchased from ICN Biomedicals (Aurora, OH) or from Accurate Chemical & Scientific Corp. (Westbury, NY). The GLT-1 cDNA in pBluescript SK- was the generous gift of Dr. B. Kanner. The GLAST cDNA was generated by reverse transcription-polymerase chain reaction with specific primers and cloned into pBluescript SK-.

Cell Culture. Astrocyte cultures were prepared from the cortices of neonatal rats (1–3 days old) as previously described (Schlag et al., 1998) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 10% Hams F-12, and 0.24% penicillin/streptomycin (10,000 U/ml penicillin, 10,000 μg/ml streptomycin). Cells were plated at a uniform density of 2.5×10^5 cells/ml (3×10^4 cells/cm^2) onto 10-cm or 12-well sterile polystyrene dishes. The cultures were maintained in a 5% CO_2 incubator at 37°C and fed with a complete medium exchange twice a week for 14 days. At 14 days in vitro, cultures reach confluency. Approximately 95% of the cells in these cultures are astrocytes based on expression of cell-specific immunohistochemical markers (for original citation, see Schlag et al., 1998). To kill contaminating oligodendrocyte precursors (A2B5-positive cells), these cultures were washed once with HEPES-buffered saline solution, and then incubated in Dulbecco's modified Eagle's medium (2 ml/10-cm dish or 500 μl/well in a 12-well plate) with A2B5 hybridoma supernatant (diluted 1:50) and rabbit complement (diluted 1:20) for 45 min at 37°C and 5% CO_2. The optimal concentration of A2B5 antibody and complement required for the complete elimination of A2B5/GLT-1 positive cells was determined in preliminary experiments. The cultures were washed 3 times with HEPES-buffered saline solution, incubated for 24 h in standard culture medium and then treated. Cells were fed with a complete medium exchange and fresh drug or vehicle every 3 to 4 days.

Cocultures of astrocytes and neurons were prepared from the cortices of day 17 prenatal rats similarly to astrocyte preparation. Cells were plated at a density of 2.5×10^5 cells/ml (3×10^4 cells/cm^2) onto 10-cm sterile polystyrene dishes coated with 100 ng/ml poly-D-lysine. The cultures were maintained in a 5% CO_2 incubator at 37°C in the same medium described above. Conditioned medium was collected as previously described (Gegelashvili et al., 1997) with a few modifications. The medium from these cultures was collected after 3 days in vitro, cleared by centrifugation (600*g* for 10 min) and supplemented with 10% FBS, 6 mM D-glucose, and 2.5 mM L-glutamine. Resulting medium was termed NCM. This medium was stored at 4°C for no more than 4 days. Astrocyte cultures free of A2B5-positive cells were treated with this medium with vehicle or drug for 3 days.

Organotypic spinal cord cultures were prepared from lumbar spinal cord of 8-day-old rat pups as described previously (Rothstein et al., 1993). In brief, lumbar spinal cords were removed and sliced into 300-μm-thick dorsal ventral sections, and five slices were placed on Millipore CM semipermeable 30-mm-diameter membrane inserts. The inserts were placed in 1 ml of culture medium in 35-mm-diameter culture wells. Culture medium consisted of 50% minimal essen-

tial medium and HEPES (25 mM), 25% heat-inactivated horse serum, and 25% Hanks' balanced salt solution (Gibco-BRL) supplemented with D-glucose (25.6 mg/ml) and glutamine (2 mM), at a final pH of 7.2. Antibiotic and antifungal agents were not used. Cultures were incubated at 37°C in a 5% CO_2-containing humidified environment. Culture medium, with or without EGF, was changed twice weekly. Cultures were treated for 14 days, starting on day 7 in-vitro.

Measurement of L-[^{3}H]Glutamate Transport. The sodium-dependent transport of L-[^{3}H]glutamate was measured as previously described (Schlag et al., 1998). Triplicate assays were performed with Na^+-containing or choline-containing buffers. Na^+-dependent transport activity was calculated as the difference in radioactivity accumulated in the presence and absence of Na^+. Data were analyzed with linear regression analysis of an Eadie-Hofstee plot.

Immunoblot Analysis. Sample preparation and Western analysis were performed as previously described (Schlag et al., 1998). Cells were lysed by sonication in a hypoosmotic buffer. After removal of an aliquot for analysis of protein, protease inhibitors were added to this whole-cell lysate. The samples were diluted in sample buffer, boiled, and frozen at −20°C. Except where noted, equal amounts of protein were loaded into each lane. Protein samples were separated by electrophoresis on SDS/10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P). Blots were probed with both an anti-actin antibody (diluted 1:5000) and either an antibody specific for GLT-1 (carboxyl terminus-directed antibody 1:10,000) or GLAST (amino terminus-directed antibody 1:5000). The development and characterization of these antibodies have been previously described (for original citations, see Sims and Robinson, 1999). Immunoblots were visualized with ECL.

The density of immunoreactive bands was quantified using Image software (NIH). In some experiments, immunoreactive bands consistent with multimers were detected (for original citation, see Schlag et al., 1998), but the predominant bands usually had an apparent molecular weight consistent with the monomer. In the present study, the lower and the higher molecular weight bands were quantified and reported. The data were also calculated and compared using just the lower molecular weight species (monomers) and the results were the same.

Organotypic cultures were sonicated in ice-cold 10 mM PBS. Lysates were then centrifuged for 5 min at 12,000*g* at 4°C. Supernatants were collected and protein determined with Coomassie Plus Reagent. Samples (5 μg of total protein) were loaded directly onto Hybond ECL nitrocellulose membrane, with a Bio-Dot SF apparatus. Blots were washed with 50 mM Tris-buffered saline (TBS) and then blocked in 1% nonfat dry milk and 0.5% Tween 20 in TBS, and probed with the GLT-1 carboxyl terminal-directed antibody diluted 1:10,000. Immunoblots were visualized with ECL. The density of the bands was quantified with ImageQuant.

Northern Analysis. Total RNA was extracted from primary astrocytes by the single-step guanidium thiocyanate-phenol-chloroform procedure as previously described (for original citations, see Schlag et al., 1998). RNA samples were separated on a 1% agarose/6.6% formaldehyde gel in 1 × 3-(*N*-morpholino)propanesulfonic acid buffer. RNA was transferred to a Hybond N+ positively charged nylon membrane and immobilized by baking at 80°C for 2 h. Membranes were prehybridized for 2 to 3 h at 65°C and hybridized for 16 to 20 h with the specific cDNA probe at 65°C. Washes were performed at 65°C with from 2 × standard saline citrate to 0.1 × standard saline citrate. Membranes were exposed to Kodak X-OMAT film for 12 to 36 h. Radioactivity was quantified with a PhosphoImager SI with the ImageQuant analysis program. Data are expressed as a ratio of transporter-specific mRNA to cyclophilin mRNA.

The *Not*I fragment of GLT-1 cDNA clone (1.4 kb), the *Pst*I-*Hinc*II fragment of GLAST cDNA clone (0.9 kb), and the *Bam*HI-*Hin*dIII fragment of rat cyclophilin cDNA (0.7 kb) were used as specific probes for the corresponding mRNAs. cDNA probes were radiolabeled with [α-^{32}P]]deoxycytidine 5′-triphosphate by random priming with Prime-it II kit (Stratagene, La Jolla, CA).

Immunocytochemistry. Primary astrocyte-enriched cultures were plated onto sterile glass coverslips coated with poly-D-lysine (50 μg/ml). For A2B5 staining, cultures were washed twice in Ham's F-12 and then incubated with undiluted A2B5 hybridoma supernatant for 30 min on ice. After two washes with PBS, the cells were incubated with goat anti-mouse fluorescein conjugate (diluted 1:50) for 30 min. These cells and all other cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After rinsing in TBS containing 50 mM Tris, 150 mM NaCl, pH 7.4, cells were incubated with TBS containing 5% normal goat serum and 0.1% Triton X-100 (TGT) for 30 min at room temperature. Cultures were incubated overnight at 4°C in TGT containing mouse monoclonal anti-GFAP (diluted 1:100) and the rabbit anti-transporter antibody (amino or carboxyl terminus-directed GLT-1 antibody diluted 1:100). After rinsing in TBS containing 0.1% Triton X-100, coverslips were incubated in TGT containing anti-mouse IgG-fluorescein and anti-rabbit IgG rhodamine conjugates (diluted 1:200) for 2 h at room temperature. According to the manufacturer's description, both secondary antibodies display minimal interspecies cross-reactivity. We have previously demonstrated that the transporter immunoreactivity is not observed with preimmune serum and is eliminated by appropriate peptides (for original citations, see Schlag et al., 1998). In addition, separate experiments omitting primary or secondary antibody were performed to test for specificity and possible cross-reaction (data not shown). To visualize cell nuclei, cultures were rinsed and then incubated in 4′,6-diamidino-2-phenylindole dihydrochloride hydrate diluted 1:500 in PBS for 10 min at room temperature. To dehydrate the tissue, coverslips were immersed in 97% ethanol for 2 min and air dried. The coverslips were mounted in Immumount and allowed to dry. Pictures were taken with an Axiophot microscope (Zeiss Instruments, Thornburg, NY).

Transient Transfection of Astrocytes with Phosphatidylinositol 3-Kinase (PI3K) Constructs. pCG-p110* and pCG-p110*Δkin constructs have been described previously (Hu et al., 1995). pCG-p110* encodes a constitutively active derivative of the catalytic subunit (p110) of PI3K under the control of a cytomegalovirus promotor. This construct is a chimera of the iSH2 domain of the p85 regulatory subunit fused to the amino terminus of the p110 catalytic subunit of PI3K. PCG-p110*Δkin is a kinase-deficient version of p110* in which the ATP-binding site of the p110 catalytic subunit is mutated. Astrocyte cultures were grown to confluency. The A2B5-positive cells were removed by complement-mediated cytolysis described above. Five milliliters of serum-free medium containing 60 μl of GenePorter transfection reagent and 10 μg of DNA was added to the cells. After a 4-h incubation at 37°C in 5% CO_2, 5 ml of medium containing 20% FBS was added and cells were incubated for an additional 24 h. The medium was replaced with medium containing 10% FBS. Cells were harvested for protein analysis 2 days later. Green fluorescent protein (GFP) expression vector pEGFP-C3 from Clontech Laboratories (Palo Alto, CA) was used to monitor the efficiency of transfection in each experiment.

Results

GLT-1 Is Expressed in A2B5-Positive Cells. In earlier studies, we and others have found that primary astrocyte-enriched cultures express low levels of GLT-1 immunoreactivity (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al., 1998). Two conditions, coculturing with neurons or treatment with cAMP analogs, induce expression of GLT-1 and cause differentiation of astrocytes in culture. A similar association of GLT-1 expression with maturation of astrocytes occurs in vivo (for review, see Sims and Robinson, 1999). Several growth factors have been implicated in controlling lineage restrictions and differentiation of glial pro-

genitor cells both in vivo and in vitro. In our initial studies, we examined the effects of a number of growth factors on GLT-1 expression in primary astrocyte-enriched cultures by Western analysis. Treatment with PDGF, EGF, and TGF-α for 7 days induced expression of GLT-1 protein in these cultures (data not shown). EGF and TGF-α changed the morphology of the astrocytes (from polygonal to stellate and process bearing), but PDGF had no apparent effect on astrocyte morphology. All three factors also caused proliferation of small process-bearing cells that sat on the astrocyte monolayer; the greatest proliferation was observed with PDGF treatment. Cells with a similar morphological appearance, whose proliferation is stimulated by PDGF, have been previously identified as bipotential glial progenitor cells characterized by expression of the ganglioside A2B5. In secondary culture and/or depending on growth conditions, these cells can differentiate into either oligodendrocytes or type-2 astrocytes (Kahn and Vellis, 1995). To investigate the possibility that these cells contribute to the expression of GLT-1 in astrocyte-enriched cultures, A2B5, GFAP, and GLT-1 expression was analyzed by immunocytochemistry with specific antibodies. In both untreated (Fig. 1A) and PDGF-treated cultures (data not shown; $n = 2$), all of these small process-bearing cells expressed A2B5. None of the A2B5-positive cells were GFAP-positive, indicating that they do not belong to astrocyte lineage (data not shown), but all A2B5-positive cells expressed GLT-1 immunoreactivity (Fig. 1A). In contrast, only background staining was detected in type-1 astrocytes (GFAP positive A2B5 negative) with antibodies against either carboxyl- (Fig. 1, A and B) or amino-terminal peptides of GLT-1 (data not shown). To confirm the expression of GLT-1 transporter in A2B5 progenitor cells, the expression of GLT-1 transporter also was examined in purified cultures of A2B5-positive cells generated by immunopanning (Grinspan et al., 1996). Immunostaining revealed the presence of GLT-1 immunoreactivity in both cell bodies and processes of these cells (data not shown). These observations suggest that A2B5-positive cells contribute to GLT-1 immunoreactivity in primary glial cultures.

To eliminate the contribution of proliferating A2B5-positive cells to total GLT-1 expression in these cultures, we adapted an antibody-based complement-mediated cytolysis procedure (for original citation, see Grinspan et al., 1996). After elimination of A2B5-positive cells, GLT-1 immunoreactivity was not detected by immunocytochemistry (Fig. 1B) or Western analysis (Fig. 2) in untreated astrocytes.

EGF Receptor Agonists Induce GLT-1 Protein Expression in Cultured Astrocytes. Confluent primary astrocyte cultures free of A2B5-positive cells were treated with mouse recombinant EGF (30 ng/ml) for 7 days starting at day 14 as described in *Experimental Procedures*. Morphological effects were evaluated by visual observation and by exami-

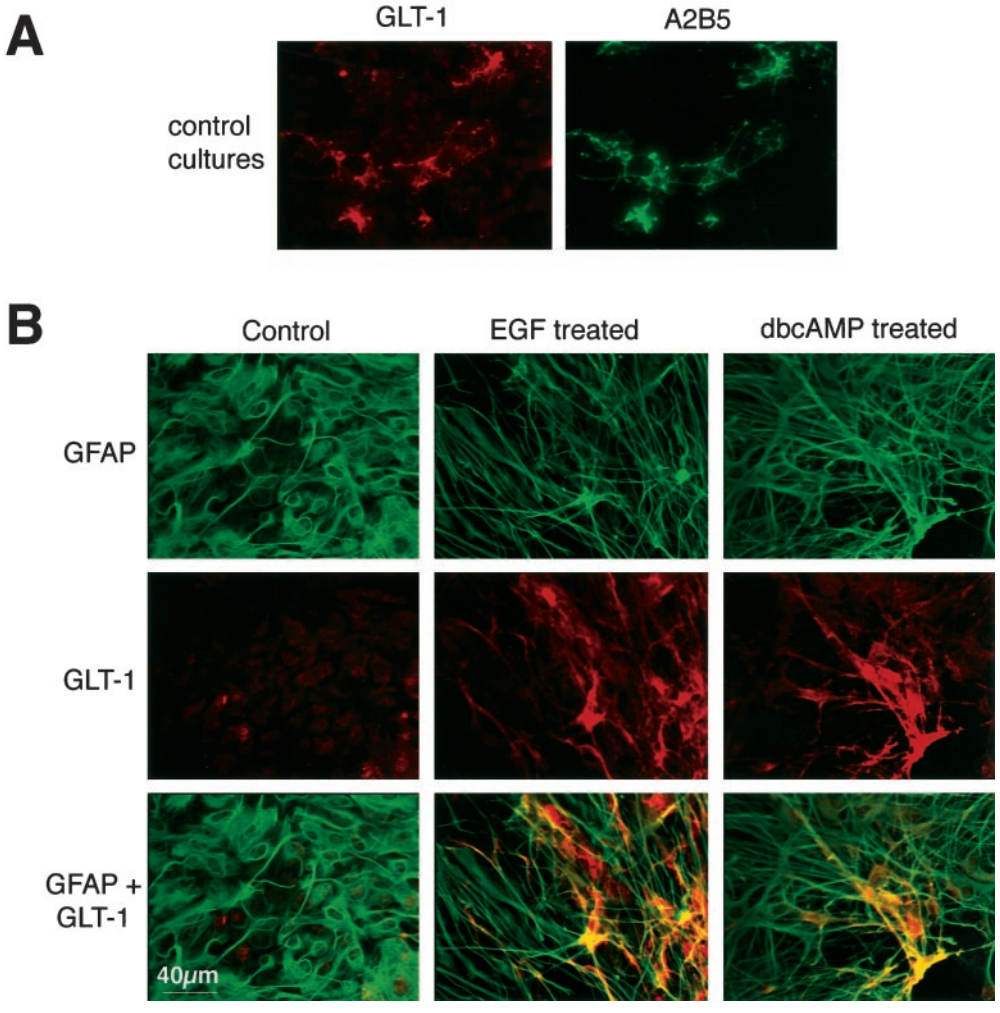

Fig. 1. Expression patterns of GLT-1 immunoreactivity in astrocyte cultures. A, double label immunocytochemical localization of GLT-1 and A2B5 in astrocyte-enriched cultures before elimination of A2B5-positive cells with complement-mediated cytolysis. In these cultures, all GLT-1 immunoreactivity colocalizes with A2B5. B, double label immunocytochemical localization of GLT-1 and GFAP in control, EGF-, and dbcAMP-treated astrocytes. After elimination of the A2B5-positive cells with complement-mediated cytolysis, the cultures were treated with vehicle, EGF (30 ng/ml), or dbcAMP (250 μM) for 7 days. This experiment was repeated at least two additional times.

nation of the pattern of GFAP immunoreactivity. EGF caused a pronounced change from a polygonal morphology to a complex process-bearing morphology similar to that observed in dbcAMP-treated cultures. The morphological effects of EGF treatment can be easily demonstrated with GFAP immunohistochemistry (Fig. 1B, top). The change in morphology accompanied an increase in GLT-1 immunoreactivity (Fig. 1B, middle). It is noteworthy that not all apparently differentiated astrocytes expressed similar levels of GLT-1 immunoreactivity. However, in both EGF- and dbcAMP-treated cultures, most of the GLT-1 immunoreactivity colocalized with GFAP (Fig. 1B, bottom), indicating up-regulation of GLT-1 expression in astrocytes.

The levels of GLT-1 expression were monitored by Western analysis and compared with the levels of transporter induced by treatment with dbcAMP (250 μM). EGF caused a pronounced increase in GLT-1 immunoreactivity comparable with that observed with dbcAMP (Fig. 2A). TGF-α, another ligand for EGFR, mimicked the effect of EGF treatment on GLT-1 expression, suggesting that synthesis of transporters can be stimulated by activation of the EGFR (Lee et al., 1995). TGF-α also caused a significant change in astrocyte morphology, comparable with the effects of dbcAMP and EGF (data not shown). To determine whether up-regulation of transporter expression is EGF-specific, other growth factors, including PDGF, NGF, bFGF, and insulin, were tested; none of these factors had a significant effect on GLT-1 expression (Fig. 2) or cell morphology. The kinetics of GLT-1 protein induction in cultures treated with EGF and TGF-α were examined and were similar to those previously observed in dbcAMP-treated cultures (Schlag et al., 1998) with only a slight increase observed after 3 days of treatment and maximal expression observed after 7 to 10 days (data not shown; $n = 3$). The effects of both EGF receptor agonists were concentration-dependent with maximal effects observed at 30 ng/ml (Fig. 3).

Previous studies revealed that expression of the other as-

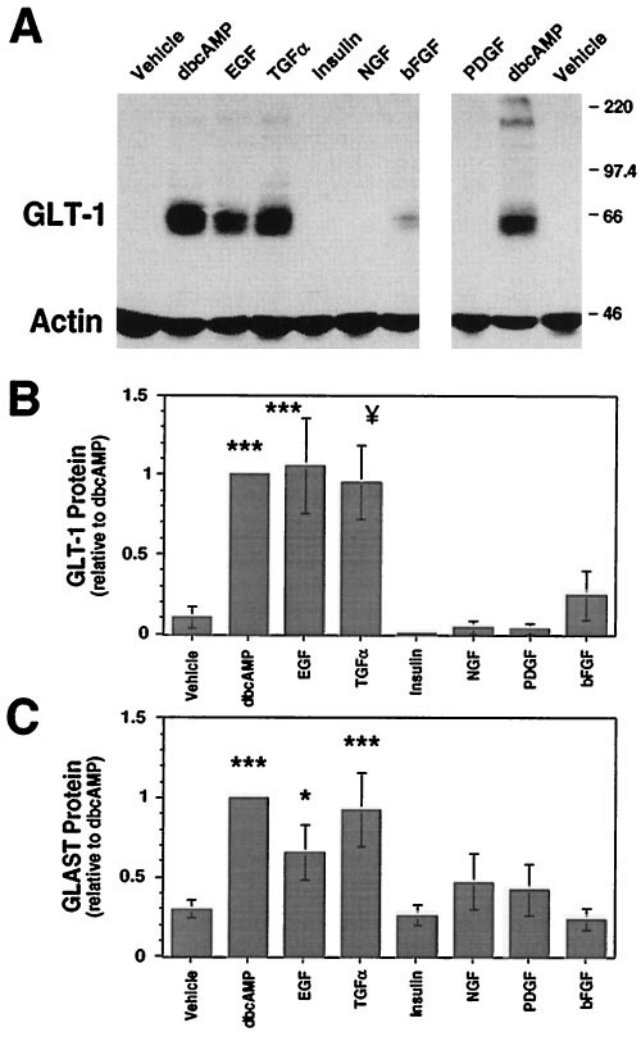

Fig. 2. Expression of glutamate transporters, GLT-1 (A and B) and GLAST (C), in primary astrocytes. A, two representative Western blots showing GLT-1 immunoreactivity and actin immunoreactivity in astrocytes. After elimination of A2B5-positive cells, astrocytes were treated with vehicle (control), dbcAMP (250 μM), EGF (30 ng/ml), TGF-α (30 ng/ml), insulin (100 μM), NGF (30 ng/ml), bFGF(100 ng/ml), or PDGF(30 ng/ml) for 7 days; 50 μg of total protein was loaded in each lane. Summaries of GLT-1 (B) and GLAST (C) expression levels from several independent experiments are shown. Data are expressed relative to the levels observed in dbcAMP-treated cultures and are means ± S.E. of three to nine independent experiments. Data were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. $^{*}P < .05$, $^{¥}P < .005$, and $^{***}P < .001$ compared with vehicle control.

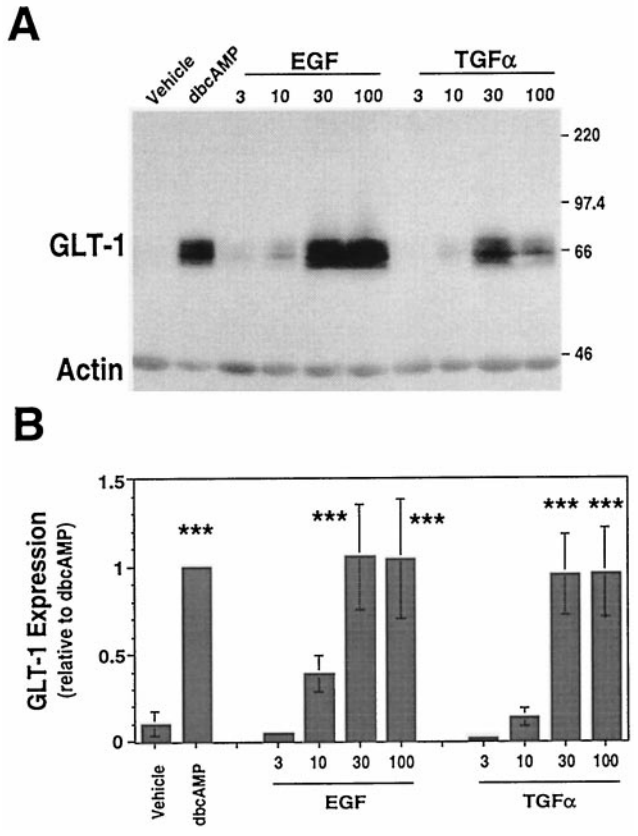

Fig. 3. Concentration-dependence of GLT-1 induction by EGF or TGF-α. A, representative Western blot showing GLT-1 immunoreactivity in cultured astrocytes treated with increasing doses of either EGF or TGF-α. Confluent cultures of primary astrocytes free of A2B5-positive cells were treated with dbcAMP (250 μM), mouse recombinant EGF, or TGF-α for 7 days. Concentrations of growth factors are in nanograms per milliliter. Fifty micrograms of total protein was loaded in each lane. B, summary of GLT-1 expression from several independent experiments. The levels of GLT-1 immunoreactivity are expressed relative to the levels observed in dbcAMP-treated cultures and are the mean ± S.E. of four to six independent experiments. Data were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. $^{***}P < .001$ compared with vehicle control.

trocytic transporter, GLAST, also is stimulated by dbcAMP treatment (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al., 1998). As was observed with GLT-1, EGF and TGF-α caused significant increases in GLAST protein levels, whereas other growth factors had no effect on GLAST expression (Fig. 2B). Coordination of GLT-1 and GLAST expression in response to dbcAMP and growth factors may suggest similarities in the regulation of both astrocytic transporters. Consistent with previous data (Schlag et al., 1998), Western analysis revealed low levels of EAAC1 and EAAT4 immunoreactivity in untreated cultures, and expression of these transporters was not affected by treatment with EGF or TGF-α ($n = 3$; data not shown).

EGF Increases Steady-State Levels of Transporter mRNAs. Regulation of protein expression by growth factors can be mediated by transcriptional as well as post-transcriptional mechanisms. To determine what mechanism underlies the increase in the levels of GLT-1 and GLAST transporters in response to growth factor treatment, the steady-state levels of GLT-1 and GLAST mRNA were analyzed in control astrocytes and in cultures treated with EGF, TGF-α, bFGF, or PDGF by Northern analysis. Hybridization with specific cDNAs revealed single mRNA bands for GLT-1 and GLAST with apparent sizes of ~11 and 3.9 kb, respectively. Control astrocytes expressed background levels of GLT-1 mRNA (Fig. 4). In astrocytes treated with either EGF or TGF-α for 7 days, GLT-1 mRNA levels were easily detected (Figs. 2 and 4). GLAST mRNA levels also were up-regulated by both EGF and TGF-α. Neither PDGF nor bFGF caused significant increases in GLT-1 or GLAST mRNA levels, consistent with the lack of their effect on protein expression. The kinetics of the change in GLT-1 and GLAST mRNA levels were similar to those for protein with a slight increase in mRNA expression observed at day 3 of treatment and maximal levels reached by day 7 (data not shown; $n = 2$). Therefore, these treatments caused parallel changes in mRNA and protein. Accumulation of GLT-1 mRNA after treatment with growth factors or dbcAMP can result from either activation of gene transcription or from an increase in mRNA stability. We considered using inhibitors of transcription to address this issue, but treatment of astrocytes with actinomycin D for >2 days killed almost all of the cells.

EGF Increases Na^+-Dependent L-[^{3}H]Glutamate Transport Activity. Na^+-dependent glutamate transport activity was examined to determine whether the increase in transporter protein expression correlated with an increase in transport activity (Fig. 5). The effects of EGF were compared with the effects of treatment with dbcAMP. As was previously observed, dbcAMP treatment increased the V_{max} value for glutamate transport (control 26.6 ± 1.5, dbcAMP-treated 40.5 ± 6.2 nmol/mg protein/min, $P < .05$, $n = 3$) but had no effect on the K_m (control 174 ± 29, dbcAMP treated 234 ± 51 μM). Increases in both the K_m and V_{max} values were observed in cultures treated with EGF ($K_m = 463 \pm 54$, $P < .005$ compared with control; $V_{max} = 65.5 \pm 7.4$, $P < .001$ compared with control, $n = 4$). The increase in V_{max} is consistent with the observed up-regulation of GLT-1 and GLAST protein. The change in K_m may be due to induction of GLT-1, which may have a lower apparent affinity for glutamate. Alternatively, it is possible that this apparent change in K_m is related to an artifact of a local drop in concentration of glutamate near the site of transport, which could be caused by the increased V_{max} (for recent discussion, see Schlag et al., 1998).

Several studies suggest that the excitatory amino acid analog dihydrokainate selectively inhibits GLT-1-mediated transport activity at concentrations <100 μM (IC_{50} value of ~50 μM). Although dihydrokainate inhibits EAAC1-mediated transport with an IC_{50} value of ~1 mM, GLAST-mediated transport activity is insensitive to inhibition (for review, see Robinson, 1999). Therefore, we examined the sensitivity of Na^+-dependent glutamate transport to inhibition by 60 μM dihydrokainate in control and EGF-treated cultures. In control cultures, dihydrokainate had no effect on transport

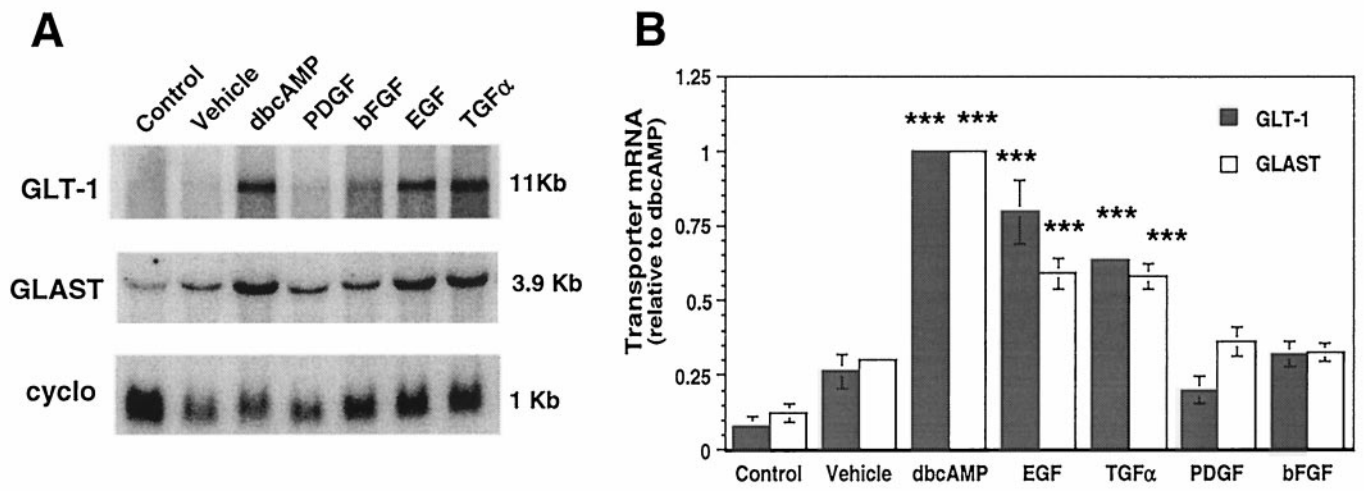

Fig. 4. Expression of GLT-1 and GLAST mRNA in astrocytes. A, representative Northern blot of GLT-1, GLAST, and cyclophilin (cyclo) mRNAs in astrocytes. After elimination of A2B5-positive cells, astrocytes were treated with vehicle, dbcAMP (250 μM), EGF (30 ng/ml), TGF-α (30 ng/ml), bFGF(100 ng/ml), or PDGF(30 ng/ml) for 7 days. Untreated cultures were harvested 24 h after the cytolysis of A2B5-positive cells (control) and 7 days later (vehicle). Twenty-five micrograms of total RNA was loaded in each lane. The data presented are from the same blot that was hybridized with GLT-1, striped, and rehybridized with GLAST and cyclophilin probes. With each probe, only a single band was observed. B, summary of four independent experiments. The levels of transporter mRNAs were normalized to cyclophilin mRNA and expressed relative to the level of corresponding mRNA in dbcAMP-treated cells. Data are means ± S.E. and were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. ***$P < .001$ compared with vehicle.

activity (1.1 ± 2.6% inhibition, $n = 5$). In parallel experiments, dihydrokainate partially inhibited transport activity in astrocyte cultures treated with EGF for 7 days (8.6 ± 2.1% inhibition, $P < .05$ compared with control, $n = 5$). This suggests that GLT-1 is functional and contributes to transport activity only in the EGF-treated cultures.

Effects of Inhibitors of Signaling Molecules on GLT-1 Induction. The biological response to a growth factor is determined by the ability of the corresponding receptor to activate specific signaling pathways. The initial step of the signaling cascades induced by EGF requires autophosphorylation of tyrosine residues on the intracellular domain of the EGFR in response to ligand binding and receptor dimerization (Yamada et al., 1997). In contrast, the effects of dbcAMP are generally attributed to activation of protein kinase A (PKA) and downstream signaling molecules. To define the signaling molecules involved, the effects of the PKA inhibitor KT5720 and the protein tyrosine kinase inhibitor genistein on the induction of GLT-1 protein were examined by Western analysis. Consistent with our previous data (Schlag et al., 1998), KT5720 blocked induction of GLT-1 protein in dbcAMP-treated astrocytes (Fig. 6, C and D). In parallel cultures treated with EGF, KT5720 had no significant effect on GLT-1 expression (Fig. 6, A and B). In contrast, genistein blocked the effect of EGF but had no significant effect in dbcAMP-treated astrocytes. Similarly, compound 56, a recently identified potent and specific inhibitor of EGFR tyrosine kinase activity (Bridges et al., 1996), selectively blocked the effects of EGF but not dbcAMP (Fig. 6). Although another tyrosine kinase inhibitor, tyrphostin A25, had no effect, others have observed that this compound is not effective with chronic incubations (Chew et al., 1994). The simplest explanation for this lack of an effect is that this compound is not stable during chronic incubations.

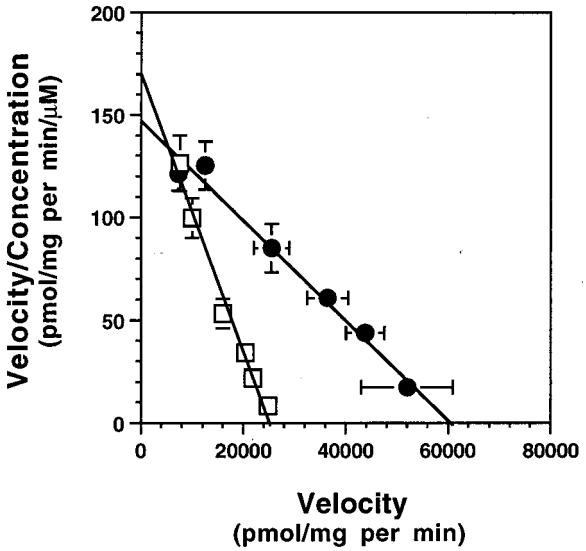

Fig. 5. Eadie-Hofstee transformation of the concentration-dependence of Na^+-dependent L-[^{3}H]glutamate transport activity in astrocyte cultures. Confluent cultures of primary astrocytes free of A2B5-positive cells were treated with vehicle or mouse recombinant EGF (30 ng/ml) for 7 days. Na^+-dependent glutamate transport activity was measured in control (□) and EGF-treated (■) cultures. In control astrocytes, the average K_m value was 174 ± 29 μM and the average V_{max} value was 26.6 ± 1.5 nmol/mg protein/min. In EGF-treated astrocytes, the average K_m value was 463 ± 54 μM ($P < .005$ compared with control); and the average V_{max} value was 65.5 ± 7.4 nmol/mg protein/min ($P < .001$ compared with control; $n = 4$).

A number of common substrates, such as phospholipase Cγ (PLCγ), PI3K, and the adaptor proteins SHC and GRB2, have been found to associate with activated tyrosine kinase receptors, including the EGFR (Yamada et al., 1997). Recruitment of the GRB2 protein mediates downstream activation of Ras. Ras can activate the Raf-MEK-mitogen-activated protein (MAP)/extracellular signal receptor-activated (Erk) kinase pathway (Egan and Weinberg, 1993) as well as PI3K (Rodrigues-Viciana et al., 1996). There are also Ras-independent pathways from EGF receptor to MAP/Erk kinase (Burgering et al., 1993). To define the signaling molecules involved in the regulation of GLT-1, the effects of cell-permeable inhibitors on EGF- and dbcAMP-induced GLT-1 expression were examined. Bis II was used to block the downstream target of PLCγ, Ca^{2+}/diacylglycerol-dependent protein kinase C (PKC). Bis II had no effect in either dbcAMP- or EGF-treated cells. Although every lot of Bis II used in the present study was tested for its ability to block acute effects of phorbol esters on EAAC1-mediated transport (for review, see Sims and Robinson, 1999), we cannot rule out the possibility that this compound may be degraded during the chronic treatments used in the present study. PD98059, a selective inhibitor of MEK (Dudley et al., 1995) partially attenuated the effects of dbcAMP, but did not block the EGF-induced increase, indicating that the MEK-MAP/Erk pathway is not involved in regulation of GLT-1 expression by EGF. The observation that PD98059 attenuated the dbcAMP-induced increase argues that the lack of inhibition in EGF-treated cells cannot be attributed to degradation of the compound or some other artifact related to the long incubations required in these experiments. Importantly, LY294002, a specific and potent inhibitor of PI3K (for original citation, see Duronio et al., 1998), blocked induction of GLT-1 protein in both EGF- and dbcAMP-treated cultures, suggesting that the function of this kinase may be essential for GLT-1 expression. Another classical inhibitor of PI3K, wortmannin, had no effect on the induction of GLT-1 (100 nM, $n = 3$; data not shown). It is likely that this lack of an effect is related to the observation that wortmannin remains effective for only ~3 to 5 h in culture medium (Kimura et al., 1994).

In several systems, $p70^{S6}$ kinase and the nuclear transcription factor NF-κB have been identified as possible downstream mediators of PI3K-dependent signaling (Béraud et al., 1999; Duronio et al., 1998). EGF also activates NF-κB in some cell types, including A-431 carcinoma, mouse embryo fibroblasts, and rat vascular smooth muscle cells, although these effects are not well studied (Sun and Carpenter, 1998). To test the involvement of these two downstream effectors of PI3K, rapamycin (an inhibitor of $p70^{S6}$ kinase) and PDTC (an inhibitor of NF-κB activation) were used (Scherck et al., 1992; Brown et al., 1995). Rapamycin had no effect on either dbcAMP- or EGF-mediated increases in GLT-1 protein, suggesting that $p70^{S6}$ kinase is not involved. PDTC inhibited both the EGF- and dbcAMP-mediated increases in GLT-1 expression. The morphology of the cells was examined to determine whether the inhibitors tested also prevented differentiation. All of the compounds that inhibited GLT-1 induction also blocked astrocyte differentiation and none of the other compounds prevented differentiation. Together, these studies suggest that both dbcAMP and EGF induce differentiation and increase GLT-1 expression in cultured astrocytes.

Because the experiments with both LY294002 and PDTC

suggested that EGF and dbcAMP increase GLT-1 expression through converging signaling pathways, the effects of a combined treatment with EGF and dbcAMP were examined (Fig. 7). In these studies, dbcAMP increased the levels of GLT-1 above those observed with maximal concentrations of EGF. Quantitation of three independent experiments revealed that the level of GLT-1 expression observed in astrocytes treated with either concentration of dbcAMP combined with EGF was within 20% of the sum of the levels observed in astrocytes treated with these reagents separately. Therefore, the effects of EGF and dbcAMP are additive, but there was no evidence of a synergistic effect when the dbcAMP was combined with maximal concentrations of EGF.

Characterization of Effects of NCM. As was previously observed, medium from mixed cultures of neurons and astrocytes (NCM) induced expression of GLT-1 in astrocyte cultures (Fig. 8). This effect has been attributed to a factor(s) (Gegelashvili et al., 1997; Schlag et al., 1998) that is secreted by either neurons or glial cells stimulated to secrete these factors in the presence of neurons. To determine whether the same signaling pathways mediate the effects of NCM, astrocyte cultures were treated with NCM for 3 days in the absence or presence of inhibitors that blocked GLT-1 induction in the EGF- or dbcAMP-treated cultures. Inhibition of PKA or the MEK-MAP/Erk pathway with KT5720 or PD98059, respectively, partially attenuated the effects of NCM. Compound 56, a selective inhibitor of EGFR tyrosine kinase activity, had no effect, but a general tyrosine kinase inhibitor, genistein, almost completely blocked the effects of NCM. As was observed for dbcAMP and EGF, the inhibitors of PI3K (LY294002) or NF-κB (PDTC) almost completely blocked the increase in GLT-1 protein induced by NCM.

Transient Transfection with Constitutively Active PI3K Induces Expression of GLT-1. To test whether activation of PI3K is sufficient to induce expression of GLT-1, primary astrocytes were transiently transfected with pCGp110* DNA encoding a constitutively active form of PI3K. A pCGp110Δkin construct that expresses a kinase-deficient version of the protein was used as a control. The efficiency of transfection was evaluated visually in parallel experiments with a pEGFP construct driving the expression of green fluorescent protein. Despite the fact that only 10 to 20% of the primary astrocytes were transfected, expression of the constitutively active PI3K caused an increase in GLT-1

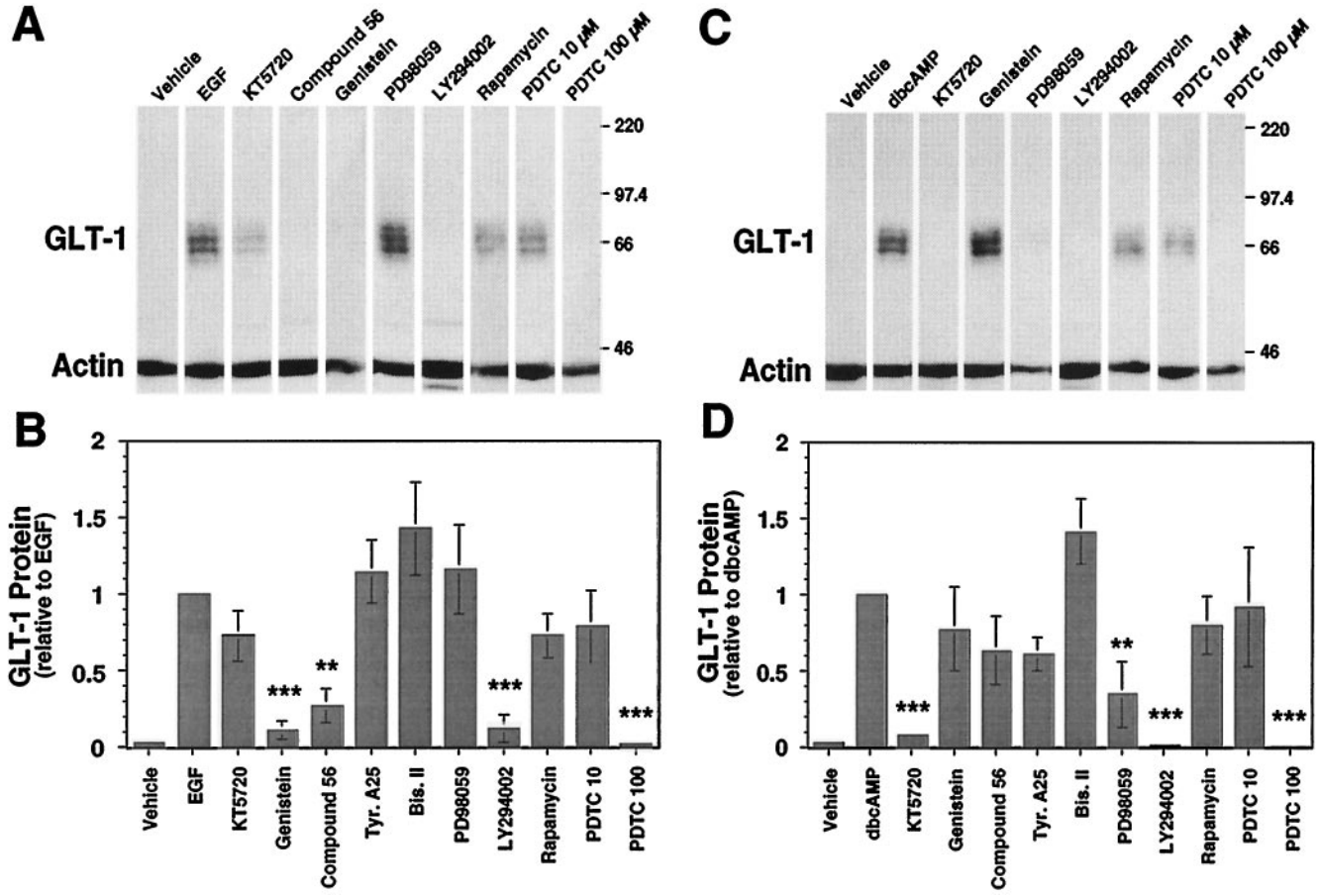

Fig. 6. Effects of inhibitors on GLT-1 protein expression in astrocytes treated with EGF (A and B) or dbcAMP (C and D). A and C, representative Western blots showing GLT-1 protein levels in astrocytes treated with EGF (A) or dbcAMP (C) with inhibitors for 7 days. After elimination of A2B5-positive cells, astrocytes were treated with vehicle, dbcAMP (250 μM), or EGF (30 ng/ml) in the absence or presence of KT5720 (5 μM), genistein (100 μM), compound 56 (1 nM), tyrphostin A25 (100 μM, Tyr.A25), Bis II (10 μM), PD98059 (100 μM), LY294002 (100 μM), rapamycin (50 nM), or PDTC (10 or 100 μM). None of these inhibitors had an effect on GLT-1 expression in control cultures (data not shown; n = 3–9). All of the lanes in A are from the same gel and all of the lanes in C are from the same gel. B and D, summaries of several independent experiments. The levels of GLT-1 immunoreactivity are expressed relative to the levels observed in EGF-treated (B) or dbcAMP-treated (D) cultures. Data are means ± S.E. of three to nine independent experiments. Data were compared by ANOVA with a Fisher Fisher Protected Least Significant Difference post hoc analysis. $^{**}P <$ .01, $^{***}P <$ .001 compared with EGF or dbcAMP.

protein expression compared with control cultures transfected with inactive PI3K or GFP (Fig. 9).

EGF Increases GLT-1 Expression in Spinal Cord Organotypic Cultures. The study with cortical astrocyte cultures revealed that both dbcAMP and EGF induce differentiation and GLT-1 expression at the same time. To examine the ability of EGF to increase GLT-1 levels in mature astrocytes, that already express basal physiological levels of GLT-1, we used the organotypic culture model (Rothstein et al., 1993). In these cultures, which have long-term neuronal survival with well preserved organotypic morphology, spinal synaptic connectivity is largely maintained, and normal astroglial/neuron interactions are preserved. The basal level of GLT-1 expression in these cultures is similar to that observed in mature rat cortex (data not shown). The cultures were treated with increasing concentrations of EGF for 2 weeks, starting on day 7 in-vitro. Low concentrations of EGF (1 and 10 ng/ml) increased GLT-1 protein by almost 2-fold (Fig. 10). Although the reason is not clear, it should be noted that the concentration-response curve from experiments in these cultures was somewhat different from that observed in astrocyte-enriched cultures. In the organotypic cultures, the maximal effect occurs at 1 ng/ml with higher concentrations causing less of an increase in GLT-1. In astrocyte cultures, the maximal effect occurs at 30 ng/ml with a comparable increase also observed at 100 ng/ml (Fig. 3).

Discussion

GLT-1 may be the predominant glutamate transporter in adult forebrain (Robinson, 1999). Expression of GLT-1 parallels maturation of astrocytes and synapse formation in neonatal brain (for review, see Sims and Robinson, 1999). In culture, astrocytes can be induced to mature with changes in morphology and antigenic properties similar to those observed in vivo. In previous studies, we and others have demonstrated that primary astrocyte-enriched cultures express low levels of GLT-1 protein and that dbcAMP increases GLT-1 expression (for original citations, see Sims and Robinson, 1999). Coculturing with neurons also induces differentiation of astrocytes and increases expression of GLT-1, and a secreted molecule contributes to this effect (Gegelashvili et al., 1997; Schlag et al., 1998). A goal of this study was to identify physiological molecules that can enhance expression of GLT-1 in astrocytes and to analyze signaling pathways involved in this regulation.

In the present study, the effects of growth factors previously implicated in differentiation of glial cells on the expression of GLT-1 were examined in cultured astrocytes. Our immunocytochemical data indicate that the low levels of GLT-1 protein detected in untreated astrocyte-enriched cultures by Western analysis are expressed not by astrocytes, but primarily by a subpopulation of A2B5-positive bipotential progenitor cells. Treatment with PDGF enhanced GLT-1 expression in these astrocyte-enriched cultures by stimulating proliferation of A2B5-positive cells, with no apparent effect on GLT-1 expression in astrocytes. In cultures free of A2B5-positive cells, GLT-1 mRNA and protein expression in astrocytes was induced by chronic treatment with EGF or TGF-α, but not by PDGF, insulin, NGF, or bFGF. Although EGF and TGF-α are astrocytic mitogens (Yamada et al., 1997), the observed increase in GLT-1 protein is not simply related to an increase in cell number because no immunoreactivity is observed in untreated astrocytes.

EGF and TGF-α are ligands for the EGFR (EGFR, also referred to as ErbB-1) (Lee et al., 1995). The EGFR belongs to a family of receptor tyrosine kinases ErbB-1 to 4 that play a major role in the regulation of CNS development. Astrocytes can express all four members of this receptor family (Vartanian et al., 1997). Signaling through EGFR is triggered by ligand binding, receptor dimerization, and tyrosine autophosphorylation, and is classically associated with activation of the Raf-MEK-MAP/Erk pathway (Yamada et al., 1997). Alternatively, it can result in activation of PLCγ that couples receptor activation with PKC signaling (Wang et al., 1998). Although the ErbB-1 receptor is not thought to interact with PI3K directly, EGF can activate this kinase indirectly through ErbB-2-dependent heterodimerization and transactivation of ErbB-3 that can mediate signaling through direct interaction with PI3K (Graus-Porta et al., 1994). These data suggest that EGF and TGF-α can activate at least three major signaling pathways, involving MAP/Erk kinase, PKC, and PI3K.

The observation that both EGF and dbcAMP induce differentiation and stimulate GLT-1 expression in astrocytes with similar kinetics suggests that common mechanisms may mediate these phenomena. To address this issue, the effects of cell-permeable inhibitors targeting PKA, EGFR tyrosine kinase, and its downstream effectors on GLT-1 expression were examined in EGF-treated, dbcAMP-treated, and control cultures. Data presented suggest that the effect of dbcAMP is mediated through PKA and the MAP/Erk kinase pathway. Activation of this pathway by dbcAMP has been demonstrated previously (Vossler et al., 1997). In contrast, induction of GLT-1 in EGF-treated cells was blocked by inhibitors that target protein tyrosine kinase activity, but not by inhibitors of the MAP/Erk kinase or PKC pathways. Because KT5720, genistein, compound 56, and PD98059 selectively blocked the effects of either EGF or dbcAMP, it seems likely that at least two specific signaling pathways are involved in the induction of GLT-1. Inhibitors of PI3K and NF-κB blocked the EGF- and dbcAMP-mediated increases in GLT-1, suggesting that activation of both PI3K and NF-κB is required for the induction of GLT-1. Although the direct involvement of the identified signaling molecules in regulation of GLT-1 expression would be the simplest explanation of the inhibitor effect, these data do not exclude the possibility that these inhibitors block GLT-1 expression nonspecifically, for example, by affecting transcription or some other cellular process critical for gene expression. In some systems, both PI3K and NF-κB are important for cell survival (Yao and Cooper, 1995; for review, see Baichwal and Baeuerle, 1997). In the present study, LY294002 and PDTC caused some cell death and reduced the total amount of protein in each plate. Because equal amounts of total protein were analyzed in each Western analysis, it is unlikely that this inhibition is simply the result of nonspecific inhibition of protein synthesis or cytotoxicity, but we cannot rule out the possibility that these inhibitors selectively killed a subpopulation of cells that express GLT-1. However, the observation that a constitutively active PI3K induces GLT-1 synthesis demonstrates that activation of PI3K is sufficient to induce expression and provides direct evidence of a role for PI3K in the regulation of GLT-1.

In this study, expression of GLT-1 was only detectable in cultures containing process bearing, stellate-shaped cells, suggesting that GLT-1 expression is associated with differentiation. Several lines of evidence indicate that EGF and/or TGF-α may contribute to astrocyte proliferation and differentiation in vivo. There is high expression of TGF-α during development and high expression of EGF receptor in astrocytes in the developing nervous system (Ferrer et al., 1996; Kornblum et al., 1997). EGF has a mitogenic effect on the astroblasts but, as revealed by clonal analysis, also promotes terminal differentiation of cells restricted to astrocytic lineage (Johe et al., 1996). Overexpression of EGFR in the ventricular zone results in early departure of EGFR-overexpressing cells from the ventricular zone, premature expression of astrocytic markers, and differentiation into astrocytes (Burrows et al., 1997). In addition there are fewer astrocytes in the cortex of mice lacking the EGFR (Sibilia et al., 1998). Together, these data suggest that EGF and/or TGF-α have a significant role in the control of astrocyte maturation in vivo.

Although EGFR may be important for GLT-1 expression in vivo, the observation that compound 56 did not block the effects of NCM suggests that EGF does not contribute to the induction of GLT-1 by NCM. Other compounds known to block signaling activated by growth factors, including genistein (tyrosine kinase), LY294002 (PI3K), and PDTC (NF-κB) almost completely blocked the effects of NCM. This suggests that some of the intracellular signaling molecules activated by EGF contribute to the effects of NCM.

It is difficult to understand why the induction of GLT-1 requires such a long exposure to either dbcAMP or growth factors because it is predicted that PKA or NF-κB activation would exert their effects on transcription within hours (Siebenlist et al., 1994; for review, see Montminy, 1997). One possible explanation is that PI3K and NF-κB control differentiation and that there is a coordinate induction of astrocytic markers, including GLT-1, after differentiation. Alternatively, this effect may be mediated by an autocrine/paracrine regulatory loop. In fact, activation of NF-κB induces expression of a variety of cellular genes, including many cell adhesion molecules, immunoreceptors, cytokines, growth factors, and other signaling molecules (Siebenlist et al., 1994).

Understanding the mechanisms that control expression of GLT-1 may be particularly important for developing new strategies to limit excitotoxic damage in chronic and acute neurodegenerative diseases. EGFR activation up-regulates expression of other astrocytic proteins important for glutamatergic transmission, including the mGluR5 subtype of metabotropic glutamate receptor (Miller et al., 1995) and glutamine synthetase, an enzyme that converts glutamate to a nontoxic amino acid glutamine (Honegger and Guentert-Lauber, 1983). We show that expression of GLT-1, the predominant glutamate transporter, also can be stimulated by EGFR activation both in astrocyte cultures and in spinal cord organotypic cultures. Such coordinated regulation of genes controlling glutamatergic transmission may serve as a mechanism of protection against excitotoxicity and may contribute to the previously documented neuroprotective effect of EGF (Yamada et al., 1997). Mice that lack EGFR exhibit progressive neurodegeneration and survive to approximately postnatal day 20 (Sibilia et al., 1998). Because this neurodegeneration occurs during the developmental stage that is associated with a rapid induction of GLT-1 protein, it is

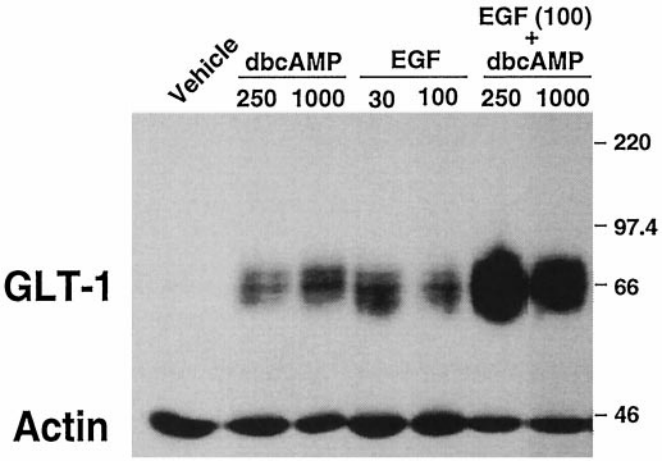

Fig. 7. Effects of combining dbcAMP and EGF on GLT-1 protein expression in astrocytes. After elimination of A2B5-positive cells, astrocytes were treated with vehicle, EGF alone (30 or 100 ng/ml), dbcAMP alone (250 or 1000 μM), or both EGF (100 ng/ml) and dbcAMP (250 or 1000 μM) for 7 days. Equal amounts of protein (50 μg) were loaded in each lane. This is a representative Western blot that has been repeated in two additional independent experiments.

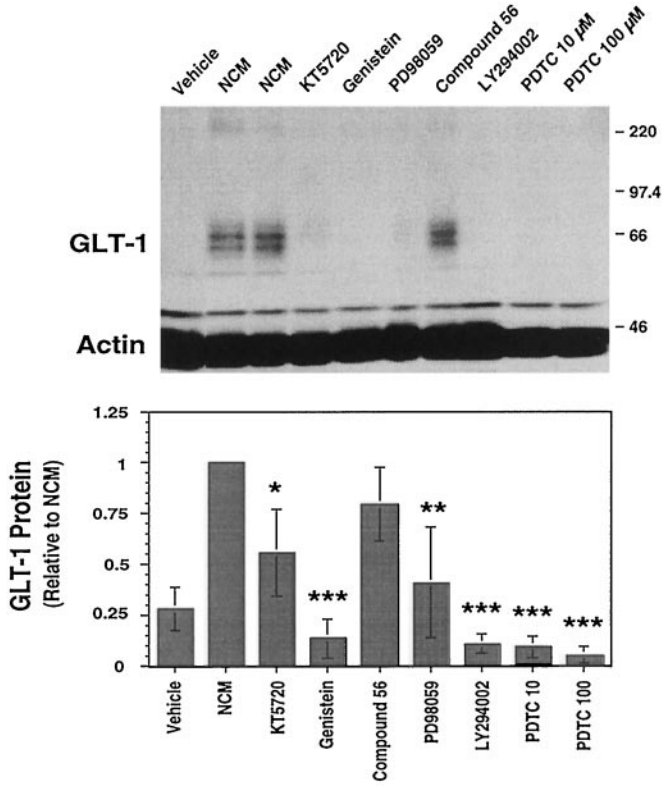

Fig. 8. Effects of inhibitors on GLT-1 protein expression in astrocytes treated with NCM. A, representative Western blot showing GLT-1 protein levels in astrocytes treated with NCM and inhibitors for 3 days. After elimination of A2B5-positive cells, astrocytes were treated with NCM in the absence or presence of KT5720 (5 μM), genistein (100 μM), compound 56 (1 nM), PD98059 (100 μM), LY294002 (100 μM), or PDTC (10 or 100 μM). B, summary of several independent experiments. Levels of GLT-1 immunoreactivity are expressed relative to the levels observed in neuron conditioned media cultures. Data are means ± S.E. of five independent experiments. Data were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. *$P < .05$, **$P < .01$, ***$P < .001$ compared with NCM.

possible that decreased expression of GLT-1 contributes to the loss of neurons. Several neurologic diseases are associated with altered expression of GLT-1. For example, a specific loss of GLT-1 is observed in the CNS regions most affected by amyotrophic lateral sclerosis. A transient decrease in GLT-1 expression is observed after neuronal injury, including ischemic insults, mechanical trauma, and deafferentation (for reviews, see Robinson, 1999; Sims and Robinson, 1999); loss of GLT-1 may exacerbate the neuronal damage. Because some of these pathological conditions are accompanied by transient decreases in EGFR and TGF-α expression (Ferrer et al., 1996), it is possible that decreased

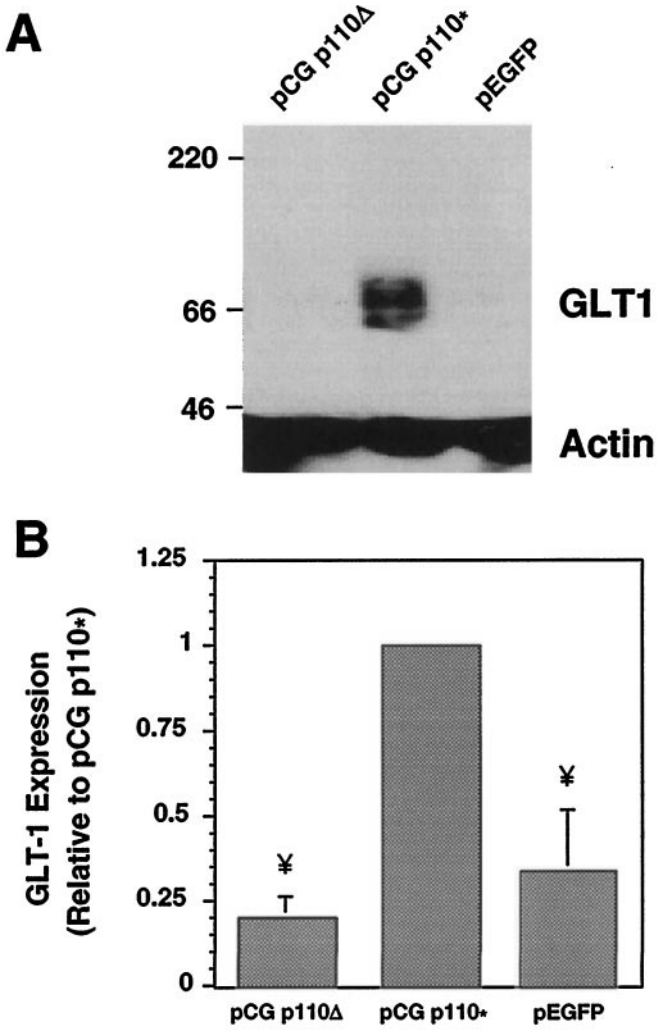

Fig. 9. Effects of a constitutively active PI3K construct on GLT-1 expression in primary astrocyte cultures. A, representative Western blot of GLT-1 immunoreactivity and actin immunoreactivity in astrocytes. After elimination of A2B5-positive cells, astrocytes were treated with 60 μl of Geneporter reagent and 10 μg of an inactive PI3K construct (pCGp110Δ), a constitutively active PI3K construct (pCGp110*), or a green fluorescence protein-expressing construct (pEGFP) in 5 ml of serum-free media. Four hours later, 5 ml of media containing 20% FBS was added. This media was replaced with fresh media containing 10% FBS 24 h later. Cells were harvested 2 days later. Fifty micrograms of protein was loaded in each lane. B, summary of GLT-1 expression from several independent experiments. The levels of GLT-1 immunoreactivity are expressed relative to the levels observed in constitutively active PI3K-transfected cultures and are means ± S.E. of five independent observations. Data were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. $^{¥}P < .001$ compared with pCG p110*.

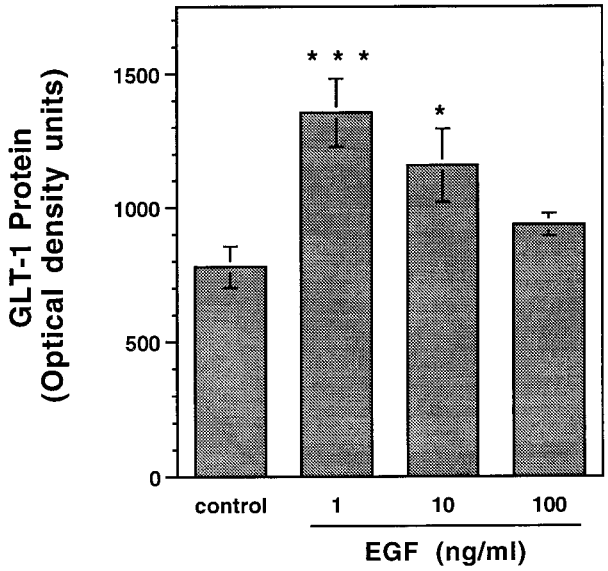

Fig. 10. Effect of EGF on GLT-1 expression in spinal cord organotypic cultures. Levels of GLT-1 immunoreactivity are expressed in random units from the densitometeric analysis of GLT-1 immunoreactivity. Data are means ± S.E. from six experiments all analyzed in parallel. Data were compared by ANOVA with a Fisher Protected Least Significant Difference post hoc analysis. $^{*}P < .05$, $^{***}P < .001$ compared with control.

EGFR activation contributes to this loss of GLT-1. Finally, it is possible that increasing the levels of GLT-1 above those observed in normal animals may reduce the susceptibility of the CNS to excitotoxic insults. Recent studies showing that transgenic animals overexpressing GLT-1 are less sensitive to seizure-induced brain damage support this possibility (Sutherland, 1998).

In summary, we demonstrate that EGF receptor agonists induce expression of the glial glutamate transporter GLT-1. This induction is accompanied by an increase in GLT-1 mRNA and transport activity and is time- and dose-dependent. By using a number of inhibitors of EGFR-dependent signaling pathways, we developed evidence that the increases in GLT-1 expression caused by dbcAMP, NCM, and EGF are dependent on PI3K and the nuclear transcription factor NF-κB. These studies suggest that independent but converging signaling pathways mediate the effects of dbcAMP and EGF. Understanding the signaling mechanisms that regulate GLT-1 expression may have important implications for developing novel strategies to limit excitotoxic brain damage.

Acknowledgments

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