Transient upregulation of the glial glutamate transporter GLAST in response to fibroblast growth factor, insulin-like growth factor and epidermal growth factor in cultured astrocytes

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SUMMARY

Although expression of the glial glutamate transporter GLAST is tightly regulated during development and under pathophysiological conditions, little is known about endogenous modulators of GLAST expression. Because growth factors are generally believed to regulate glial functions, we addressed their possible contribution to GLAST regulation in cultured rat astrocytes. Of the six growth factors tested (basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), insulin, platelet-derived growth factor, and hepatocyte growth factor), bFGF, IGF-1 and EGF enhanced [^{3}H]glutamate transport activity in a concentration-dependent manner. These effects were accompanied by an increase in the V_{max} value for transport activity and in GLAST protein and mRNA levels, which suggests that GLAST expression is transcriptionally regulated by the growth factors. Interestingly, the effects reached a peak after 36 hours of exposure to growth factors, and rapidly returned to baseline by 48 hours. A combination of IGF-1 with either bFGF or EGF showed an additive effect on the glutamate uptake activity, but a combination of bFGF and EGF did not. Pharmacological blockade of protein kinase C inhibited the effects of IGF-1 and EGF, but not bFGF. By contrast, genistein, an inhibitor of tyrosine kinases, blocked the effects of bFGF and EGF without affecting the effect of IGF-1. These results suggest that the growth factors activate different signaling pathways for GLAST upregulation. The present study may indicate a novel regulatory system of glial glutamate transporters.

Key words: Astrocyte, GLT-1, Basic fibroblast growth factor, Epidermal growth factor, Insulin, Platelet-derived growth factor, Hepatocyte growth factor

INTRODUCTION

Glutamate is the major excitatory neurotransmitter of the mammalian central nervous system (Fonnum, 1984). Extracellular glutamate is normally kept at low levels by Na^+-dependent active transport into glial cells and neurons (Nichols and Attwell, 1990). Molecular cloning led to the isolation of cDNAs for three high-affinity glutamate transporter subtypes in nonhuman systems: GLAST (Storck et al., 1992), GLT-1 (Pines et al., 1992), and EAAC1 (Kanai and Hediger, 1992). Human homologs of three transporters EAAT1, EAAT2 and EAAT3 have also been cloned, as well as two additional transporters EAAT4 and EAAT5 (Arriza et al., 1997; Fairman et al., 1995). Immunohistochemical studies indicated that EAAT4 is expressed in the Purkinje cells (neurons) of the cerebellum (Yamada et al., 1996; Furuta et al., 1997a), that EAAC1 is expressed in both neurons (Rothstein et al., 1994) and glial cells (Conti et al., 1998; Kugler and Schmitt, 1999) and that GLAST and GLT-1 proteins are expressed only in glial cells in the adult brain and spinal cord (Lehre et al., 1995; Schmitt et al., 1996; Schmitt et al., 1997). GLT-1 is expressed in neurons in the retina (Rauen et al., 1996), in culture (Mennerick et al., 1998), during development (Northington et al., 1998, Yamada et al., 1998) and after hypoxia-ischemia (Martin et al., 1997). Several lines of evidence using antisense oligonucleotides and targeted gene disruption showed that GLAST and GLT-1 play a pivotal role in maintaining low extracellular concentrations of glutamate, thereby protecting neurons against excitotoxicity in vivo (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998). By contrast, neuronal transporter EAAC1-deficient mice develop no neurodegeneration (Peghini et al., 1997).

Recent observations indicated different developmental patterns of GLT-1 and GLAST expression (Shibata et al., 1996; Sutherland et al., 1996; Bar-Peled et al., 1997; Furuta et al., 1997b; Ullensvang et al., 1997). GLAST expression diminishes following the completion of cell migration during embryogenesis, whereas GLT-1 increases progressively to adult levels during postnatal maturation. Pathophysiological study shows that GLT-1 mRNA levels are lowered in postischemic rat hippocampus, suggesting a possible mechanism for the decreased clearance of glutamate in

ischemia models (Torp et al., 1995). Despite such dynamic changes in the expression of the glutamate transporters, little is known about mechanisms underlying these patterns of expression. When immature astrocytes bearing only GLAST are cocultured with neurons or treated with cAMP analogs, they express GLT-1 with the augmented expression of GLAST (Swanson et al., 1997; Schlag et al., 1998). Fimbria-fornix or corticostriatal lesion induces a decrease in immunoreactivity for GLAST as well as GLT-1 within the hippocampus and striatum (Ginsberg et al., 1995). These results suggest that neuronal factors are involved in the induction of GLT-1 and GLAST expression. However, very few endogenous molecules have so far been identified as intercellular regulators of the glutamate transporter.

Of extracellular soluble factors derived from neurons, growth factors are well known to modulate the morphology and functions of astroglial cells. Zelenaia et al. recently showed that epidermal growth factor (EGF) induces GLT-1 expression in immature astrocytes (Zelenaia et al., 2000). Therefore, we have focused the present study on the effects of growth factors on GLAST expression. Using primary astrocyte cultures prepared from rat cerebral cortex, we demonstrate that, of the six growth factors tested (basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), EGF, insulin, platelet-derived growth factor AB (PDGF-AB), and hepatocyte growth factor (HGF)), bFGF, IGF-1 and EGF enhance the transcriptional level of GLAST in a concentration- and time-dependent manner.

MATERIALS AND METHODS

Primary cortical astrocyte cultures

Two-day-old Wistar rats were deeply anesthetized by hypothermia, their brains were aseptically removed, and the cerebral cortices were dissected out under stereomicroscopic control. After removal of the meninges, the tissues were dissociated by trypsinization and trituration. Cells were suspended in a modified Eagle's medium supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% fetal bovine serum, and plated on uncoated 75 cm^2 flasks at an approximate density of 50,000 cells/cm^2. The cultures were maintained in humidified, 5% CO_2 incubator at 37°C. The medium was changed 24 hours after plating and then every 3-4 days. The cells were grown and became confluent after 10-14 days. At this point, more than 90% of the adherent cells exhibited the flattened, polygonal appearance typical of type I astrocytes. Non-astrocytes were detached from the flasks by shaking, and removed by changing the medium. The remaining cells were dissociated by trypsinization (0.1% trypsin-0.04% EGTA) and plated on uncoated 24-well plates at a density of 10,000 cells/cm^2. The cells became confluent again 10-14 days after the plating. Experiments were initiated 24 hours after the medium was switched to serum-free medium.

Glutamate uptake

The culture media were replaced with a modified Hanks' balanced salt solution (HBSS) containing 2 mM glucose (pH 7.2). After a 30 minute preincubation, each culture received treatment with 0.025 μCi/ml L-[^{3}H]-glutamate (Amersham Pharmacia Biotech, Uppsala, Sweden) and unlabeled glutamate to achieve final glutamate concentrations in the range of 2 μM to 200 μM. Uptake was terminated after 7-minute incubation at 37°C by three washes in ice-cold HBSS, immediately followed by cell lysis in 1 N NaOH. Aliquots were taken for scintillation counting and for Lowry's protein assay (Lowry et al., 1951) using BSA standards. Blanks prepared from osmotically lysed cells revealed that glutamate binding showed <0.3% of the ^{3}H accumulation.

Immunoblot analysis

Affinity-purified polyclonal antibodies to GLAST and GLT-1 were generous gifts from K. Tanaka (Tokyo Medical and Dental University). The specificity of these antibodies was previously reported (Shibata et al., 1997; Yamada et al., 1998).

Cultures were washed twice with cold phosphate-buffered saline (PBS), plates were scraped, and cells were suspended in 0.5 mM EDTA-PBS. The suspensions were centrifuged at 10,500 ***g*** at 4°C for 5 minutes. The pellet was resuspended in buffer containing protease inhibitors (25 μg/ml leupeptin, 25 μg/ml pepstatin, 1.25 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA) and homogenated on ice. The suspensions were then centrifuged at 300 ***g*** at 4°C for 10 minutes, and protein solutions were obtained. Proteins were denatured by heating at 70°C in 5% 2-mercaptoethanol for 20 minutes. Stacking gels (5% acrylamide) were loaded with 3 μg protein and molecular weight markers, and run at 50 V for 1 hour, then run through a 11% acrylamide gel at 100 V for 2 hours. Proteins were electrophoretically transferred to a polyvinylidenefluoride membrane (Millipore, Bedford, MA) at 80 mA for 15 hours. The membranes were stored in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat milk for 1 hour. After three washes, primary antibodies (anti-GLAST antibody 1 μg/ml, anti-GLT-1 antibody 0.5 μg/ml) were applied in Tris-buffered saline/0.1% Tween-20 (TBS-T) containing 5% nonfat milk for 1 hour at room temperature. Excess primary antibody was removed with three additional washes, and the membranes were incubated with an alkali phosphatase-conjugated anti-rabbit secondary antibody in TBS-T containing 5% nonfat milk at room temperature for 1 hour. Antibody binding was detected by an ECF western blotting detection kit (Amersham).

Immunohistochemistry

Cultures were washed twice in PBS, and fixed with 4% paraformaldehyde at room temperature for 10 minutes. After two rinses, cells were treated with 0.1% Triton X-100 at room temperature for 30 minutes. Cultures were incubated with rabbit polyclonal antibodies for GLAST (1:1000) at 4°C overnight. After being washed in PBS, coverslips were incubated in PBS containing 10 μg/ml propidium iodide and anti-rabbit IgG-FITC conjugates (1:1000) at room temperature for 4 hours. Confocal imaging was carried out with a laser scanning confocal system Micro Radiance (Biorad, Herculeus, CA) equipped with an inverted microscope ECLIPSE TE300 (Nikon, Tokyo, Japan), an argon ion laser and a host computer system. All imaging and processing operations were performed with Laser Sharp Acquisition (Biorad) and Laser Sharp Processing (Biorad), respectively. To observe propidium iodide and FITC fluorescence, the cultures were illuminated with an excitation wavelengths of 488 and 514 nm, and the fluorescence images were obtained through a 500- and 570-nm band-pass filter, respectively.

Preparation of cDNA probe

Antisense oligonucleotides for GLAST and GLT-1were prepared for northern blots. Reverse transcription of mRNA to generate cDNA was accomplished using reverse transcriptase and random hexamer (Superscript Amplification System, Life Technology, Grand Island, NY). A total of 2-4 μl of rat brain cDNA was used for PCR amplification in the presence of 2.5 U Taq DNA polymerase (Takara, Otsu, Japan) and specific primers. Sequences of the primers were 5′-CTCACTGACTGTGTTTGGTG-3′ and 5′-GAGGTGCCACCAGA-ACTTTC-3′ (457 bp product size) for GLT-1 and 5′-ATGCCTTTG-TGCTACTCACC-3′ and 5′-GTGTTTCGTTGGCCTGGATG-3′ (460 bp) for GLAST. Thermal cycling was performed as follows: one cycle at 94°C (30 seconds), then 35 cycles at 94°C (10 sec), 55°C (30 seconds), and 72°C (30 seconds), followed by 72°C (5 minutes) for final extension. Amplification without templates was used as a

negative control. The PCR products were separated by electrophoresis on 1% agarose gels, and then stained with ethidium bromide. The bands were photographed under UV light. The PCR-amplified products were subcloned into the TA cloning vector (Invitrogen, San Diego, CA) and used for transformation of INVαF competent cells. Positive transformations containing plasmids with inserts were selected by growing bacteria on LB agar plates containing 50 μg/ml ampicillin and 2% X-gal. Plasmid DNA was isolated from minicultures and digested with *Eco*RI. Plasmids containing the PCR products were then sequenced.

Northern blot analysis

Astrocytes of 14 flasks (75 cm^2) were pooled to obtain about 2 μg mRNA for each group. mRNAs were isolated from cultured astrocytes by oligo-dT selection using the mRNA purification kit (Qiagen, Valencia, CA). The mRNA samples and RNA size markers (BRL, Grand Island, NY) were electrophoresed on 2.2 M formaldehyde denaturing 1% agarose gel and blotted onto a nitrocellulose membrane. The membrane was crosslinked in an UVStratalinker 1800 (Stratagene, La Jolla, CA). Antisense oligonucleotides prepared as described above were labeled with [^{32}P] and hybridized to each membrane at 42°C for 15 hours in buffers consisting of 50% deionized formamide, 1% sodium dodecyl sulfate, 4× standard saline phosphate EDTA, 2× Denhardt's solution, and 10 μg salmon sperm DNA. After hybridization, the membranes were washed with increasing stringency at 37-55°C, and autoradiographs were developed with Kodak Biomax MS film (Eastman Kodak, Rochester, NY). After stripping, the membranes were hybridized to a labeled GAPDH probe to produce second autoradiograph.

Statistical analyses

Statistical analyses were performed with Student's *t*-test for comparisons between two groups or Tukey's test following one-way ANOVA for multiple comparisons. All results are expressed as means±s.e.m.

RESULTS

Growth factors enhance L-[^{3}H]glutamate transport activity

Glutamate transport activity of cultured astroglial cells was measured as uptake activity of L-[^{3}H]glutamate. Baseline uptake activity was completely abolished in Na^+-free medium, and also blocked by the non-selective inhibitor of high-affinity glutamate transporters D,L-threo-β-hydroxyaspartate (THA) in a concentration-dependent manner (Fig. 1A). These data indicate that the uptake activity was mediated by Na^+-dependent secondary active transport via glutamate transporters. The uptake was unaffected by the selective GLT-1 inhibitor dihydrokainic acid (DHK) (Arriza et al., 1994; Robinson, 1999), even at high concentrations (~1 mM) (Fig. 1A), which suggests that GLAST is a predominant transporter in our cultures. Thus, we consider that this culture system is useful in investigating the molecular behavior of GLAST.

The initial set of experiments aimed to determine whether growth factors modulate the high-affinity glutamate uptake system. When 1-10 ng/ml bFGF was applied continuously for 24 hours, polygonal astrocytes were morphologically changed into a process-bearing phenotype and showed a significant increase in the rate of glutamate uptake in a concentration-dependent manner (Fig. 1B). This enhancement was efficiently blocked by THA, but not by DHK (Fig. 1B), which suggests that the facilitatory effect of bFGF is due to the enhanced

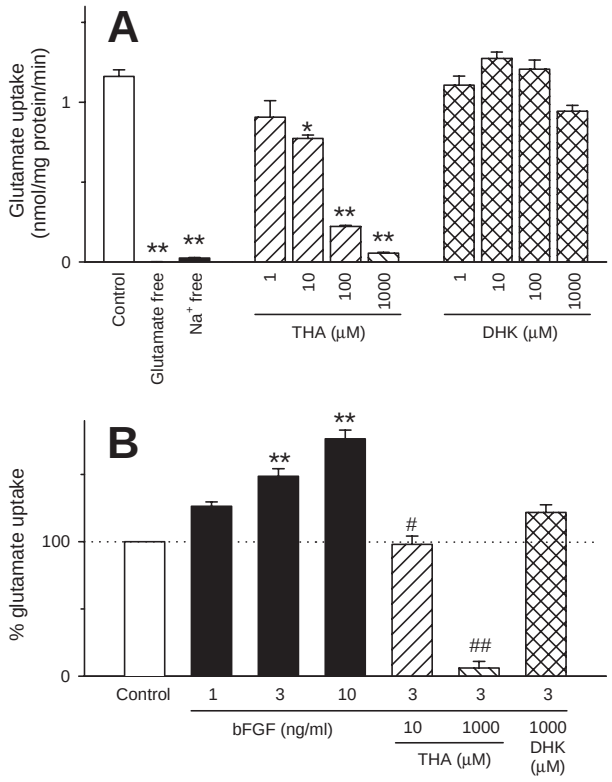

Fig. 1. bFGF enhances THA-sensitive glutamate uptake activity in astrocyte cultures. (A) [^{3}H]glutamate uptake activity of confluent astrocyte cultures was measured in [^{3}H]glutamate-free or Na^+-free medium or in the presence of THA, a non-selective inhibitor of glutamate transporters, or DHK, a selective inhibitor of GLT-1. (B) [^{3}H]glutamate uptake activity in the absence or presence of these inhibitors was measured in the astrocytes treated with bFGF for 24 hours. The relative activity is expressed as a percentage of that in intact astrocytes (Control). bFGF enhanced THA-sensitive glutamate transport activity in a concentration-dependent manner. (*<0.05; **p<0.01 vs control; $^{\#}p$<0.05; $^{\#\#}p$<0.01 vs 3 ng/ml bFGF, Tukey's test following ANOVA). Data are means±s.e.m. of 4-8 cases.

GLAST activity but not a result of the emergence of GLT-1 activity.

Similar facilitatory effects were observed when either IGF-1 or EGF was applied for 24 hours (Fig. 2). However, no apparent effect was obtained for treatment with insulin, PDGF-AB or HGF. Taken together, these results suggest that some, but not all, growth factors promote the glutamate transporter activity in cultured cortical astrocytes.

Growth factor-induced GLAST expression

Eadie-Hofstee analysis revealed that bFGF, IGF-1 or EGF significantly increased the V_{max} value for transport activity, suggesting that the effects of the growth factors are accompanied by an increase in the protein level of the glutamate transporter (Fig. 3). Consistent with this is an observation that no significant change in glutamate uptake was detected in astrocytes that had received brief treatments with growth factors for 0.5 to 12 hours (data not shown). The slow effects imply an involvement of de novo protein synthesis.

To address this possibility, we investigated the effects of pharmacological blockade of protein synthesis. The facilitatory

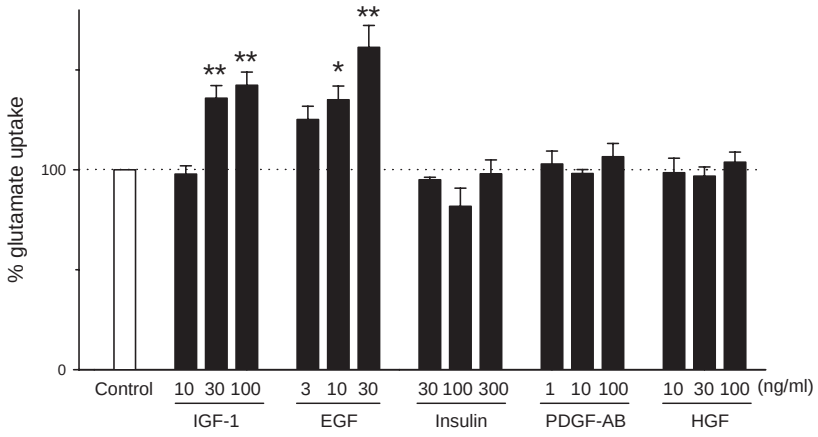

Fig. 2. Effects of growth factors on glutamate uptake activity in astrocyte cultures. Astrocytes were treated with IGF-1, EGF, insulin, PDGF-AB or HGF for 24 hours, and the glutamate uptake activity was measured. The relative activity is expressed as a percentage of control levels. Of these growth factors, IGF-1 and EGF induced an increase in the uptake activity in a concentration-dependent manner. (*p<0.05; **p<0.01 vs control, Tukey's test following ANOVA). Data are means±s.e.m. of 4-6 cultures.

effects of the growth factors were completely blocked by the translational inhibitor cycloheximide while the baseline uptake activity was unaffected by the same dose of this drug (Fig. 4A).

Western blot analysis showed that bFGF, IGF-1 and EGF induced an increase in GLAST levels (Fig. 5Aa). GLT-1 immunoreactivity was virtually undetectable in control or growth factor-treated astrocytes, whereas it was evident in adult rat brain (Fig. 5Ab). These results strongly support the hypothesis that the facilitatory effects of growth factors are entirely due to an increase in total GLAST amount. Thus, the GLAST expression was analyzed by immunohistochemistry with anti-GLAST antibody (Fig. 5B). GLAST was localized throughout the somata except for the cell nuclei in astrocytes. bFGF enhanced the immunoreactivity. But, it was unlikely to alter the subcellular localization of GLAST.

The transcriptional inhibitor actinomycin D (1 μM) completely blocked the effects of all three growth factors without affecting the baseline uptake activity (Fig. 4B), which suggests that de novo transcription is also required for the GLAST upregulation. Indeed, northern blot analysis revealed that bFGF induced an increase in GLAST mRNA level. However, GLT-1 mRNA was undetected in untreated or bFGF-treated cultures (data not shown). Thus, the growth factors are likely to modulate GLAST expression at transcriptional levels.

Transient GLAST induction following treatment with growth factors

We assessed the time course of the facilitatory effects of growth factors. When bFGF was continuously applied to astrocytes, the increase in glutamate uptake activity was observed after 24-42 hours. The effect reached a peak after 36 hours, and rapidly returned to baseline by 48 hours (Fig. 6A). The effect of IGF-1 and EGF also returned to basal levels after 48 hours (mean percent of baseline: 106.9±10.4% and 93.5±4.5% at 48 hours, respectively, n=4). These results suggest that these growth factors exert their effects within a narrow time window.

PCR products for GLAST were obtained at the predicted size by using the set of primers designed for GLAST. Twenty-five cycle RT-PCR was employed here because our preliminary experiment showed that the optical density was linear with PCR cycles (up to 30 cycles). GLAST mRNA was increased after 18 to 36 hours of bFGF treatment, and the maximal effect was given at 24 or 30 hours (Fig. 6B). The increase in GLAST mRNA levels slightly proceeded the change in glutamate uptake activity. This time lag may reflect a translational process for GLAST expression.

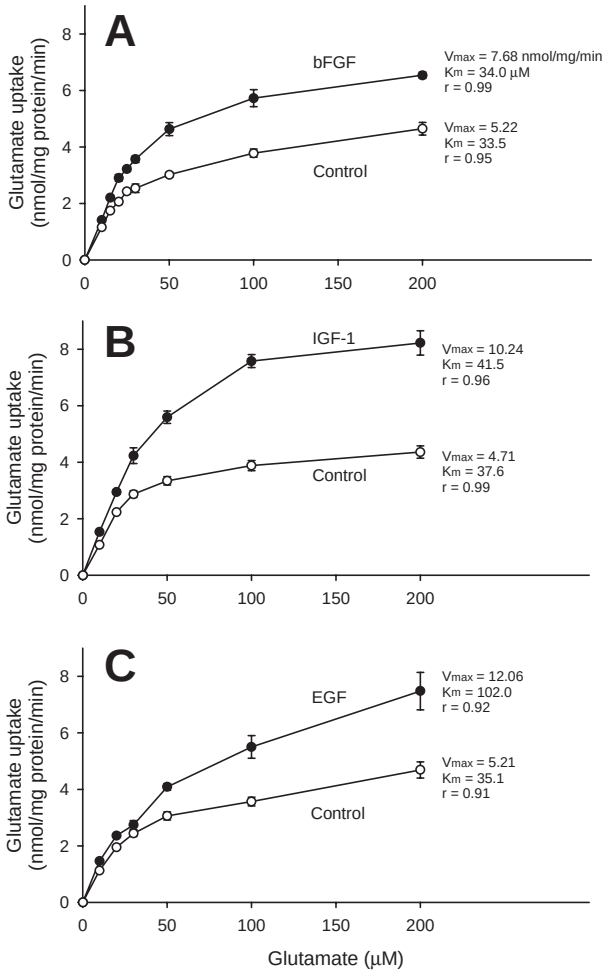

Fig. 3. Analysis of the glutamate transport activity of growth factor-treated astrocytes. A concentration dependence of glutamate transport activity was assessed in the astrocytes treated with 3 ng/ml bFGF (A), 100 ng/ml IGF-1 (B), and 30 ng/ml EGF (C) for 24 hours. The V_{max} and K_m values were calculated by a linear regression analysis of an Eadie-Hofstee transformation. All growth factors significantly enhanced the V_{max} value for transport activity (P<0.001, Student's t-test). Data are means±s.e.m. of 4-12 cultures.

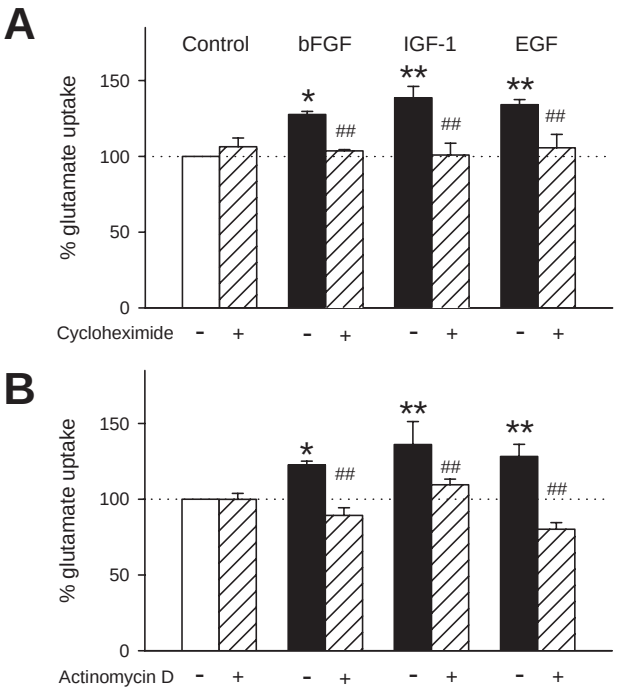

Fig. 4. Transcriptional and translational dependency of the effects of growth factors. In the presence of 1 μM actinomycin D (A) or 1 μM cycloheximide (B), astrocytes were treated with 3 ng/ml bFGF, 100 ng/ml IGF-1, or 30 ng/ml EGF for 24 hours. The facilitatory effects of the growth factors on glutamate transport activity were completely blocked by either the translational inhibitor cycloheximide or the transcriptional inhibitor actinomycin D. (*p<0.05; **p<0.01 vs control; $^{\#\#}p$<0.01 vs the corresponding growth factor, Tukey's test following ANOVA). Data represent means±s.e.m. of 4 cultures.

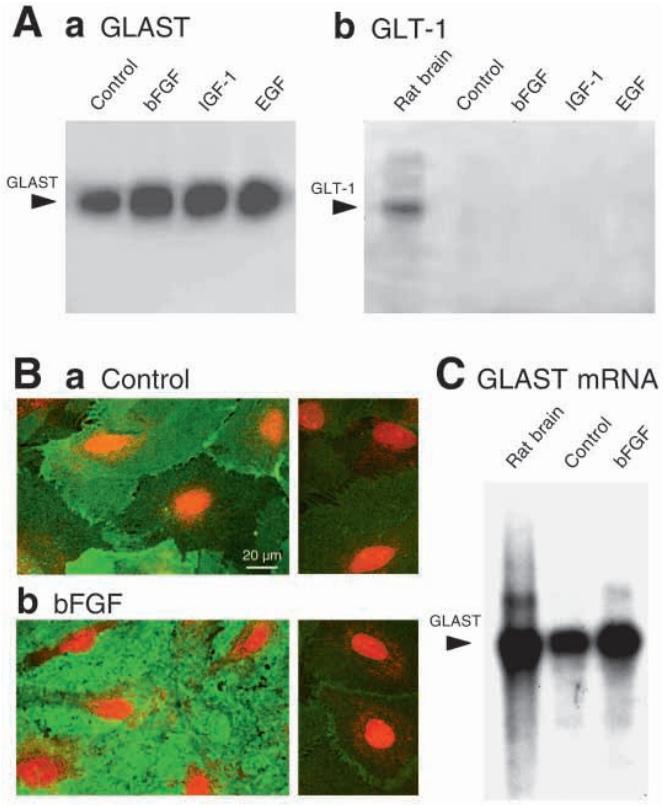

Fig. 5. Growth factors increase the expression level of GLAST. (A) Western blot analysis of the glutamate transporter GLAST (a) or GLT-1 (b) was performed for the rat whole brain or the cortical astrocytes cultured with either 3 ng/ml bFGF, 100 ng/ml IGF-1 or 30 ng/ml EGF for 36 hours. Anti-GLAST or GLT-1 antibody recognized a protein with a molecular weight of ~60 kDa. GLAST expression was increased by the incubation in the presence of bFGF, IGF-1 or EGF. GLT-1 expression was virtually undetected in the astrocyte cultures. (B) Immunohistochemical localization of GLAST (green) in the astrocytes cultured in the absence (a) or presence (b) of 3 ng/ml bFGF for 36 hours (left panels). Propidium iodide (red) was used for the counterstaining. Right panels show a nonspecific staining of the secondary IgG-FITC in the absence of anti-GLAST antibody. The result confirmed that bFGF enhanced the GLAST protein levels. (C) Northern blots were performed by using mRNA isolated from the rat whole brain, or untreated (Control) and 3 ng/ml bFGF-treated astrocytes. Treatment with bFGF for 24 hours induced an increase of GLAST mRNA levels.

Growth factors activate different signaling pathways for GLAST upregulation

To determine whether bFGF, IGF-1 and EGF share a common signaling pathway for the GLAST induction, we investigated the effect of various combinations of the growth factors on glutamate uptake activity (Fig. 7). In this series of experiments, bFGF, IGF-1 and EGF were applied at concentrations of 10 ng/ml, 100 ng/ml and 30 ng/ml, respectively, because these high concentrations rendered the maximal effect on the glutamate uptake (data not shown). A combination of bFGF and IGF-1 caused further increase in the maximal activity of glutamate uptake, compared with treatment with bFGF or IGF-1 alone. Likewise, a combination of IGF-1 and EGF induced an additive increase in the glutamate uptake. However, a combination of bFGF and EGF did not show such an additive effect. The effect of a mixture of all three growth factors was similar to that of a combination of IGF-1 with either bFGF or EGF, whereas it was significantly larger than that of a combination of bFGF and EGF. The data suggest that the effects of these growth factors are mediated, at least in part, by different signaling mechanisms. Therefore, we finally performed pharmacological investigations on the growth factor-mediated GLAST induction (Table 1).

Because growth factor receptors are known to activate several types of tyrosine kinase cascades (Leof, 2000), the possible involvement of tyrosine kinase in the GLAST regulation was assessed by examining the effect of two distinct inhibitors of tyrosine kinase, herbimycin A and genistein. Herbimycin A inhibited the effects of bFGF, IGF-1 and EGF. By contrast, genistein blocked the effect of bFGF and EGF, but not IGF-1. The data indicate that tyrosine kinase cascades are required for all three growth factors to regulate GLAST expression, whereas the genistein-sensitive tyrosine kinase pathway is not essential for IGF-1.

Growth factors are also known to stimulate the Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphatidyl inositol 3-kinase (PI3K) pathway (Leof, 2000). Indeed, *N*-acetyl-S-farnesyl-L-cysteine (AFC), an inhibitor of Ras, significantly abrogated the effects of bFGF, IGF-1 and EGF. Similarly, either the inhibitor of MAPK kinase PD98059 or the inhibitor of PI3K wortmannin blocked the effects of all three growth factors. Therefore, bFGF, IGF-1 and EGF may

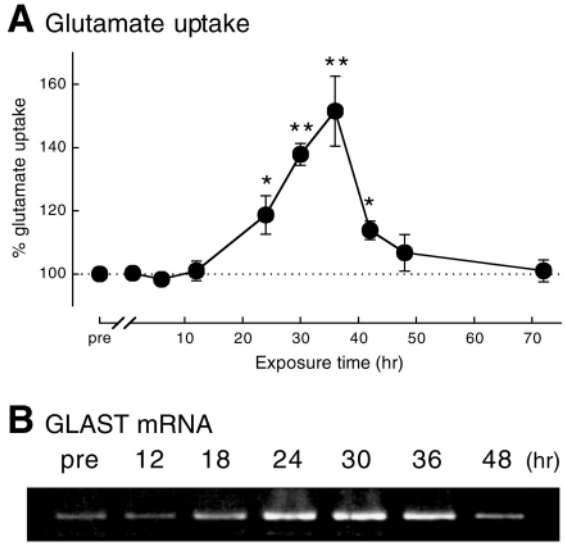

Fig. 6. Transient upregulation of GLAST expression following bFGF treatment. Time course of changes in glutamate uptake (A) and GLAST mRNA (B) was assessed in cultured astrocytes treated with 3 ng/ml bFGF. (A) The level of glutamate uptake is expressed relative to the uptake rate of untreated astrocytes (pre). The bFGF effect reached a peak after 36 hours, and rapidly returned to baseline by 48 hours. (*p<0.05; **p<0.01 vs pre, Tukey's test following ANOVA). Data represent means±s.e.m. of 4 cultures.
(B) A representative northern blot of GLAST mRNA. Numbers above the lanes indicate the duration of bFGF treatment. An increase in the mRNA levels was observed at 18 to 36 hours.

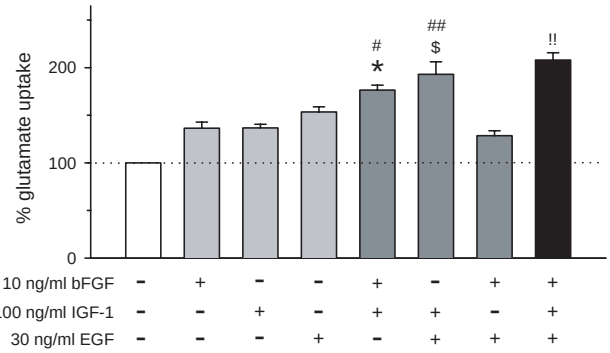

Fig. 7. Additive effects of growth factor combinations on glutamate uptake in cultured astrocytes. Astrocytes were treated with each growth factor alone or together with others for 36 hours.
A combination of IGF-1 with either bFGF or EGF showed an additive effect on glutamate uptake activity, but a combination of bFGF and EGF did not. (*p<0.05 vs bFGF alone; $^{\#}p$<0.05; $^{\#\#}p$<0.01 vs IGF-1 alone; $^{\$}p$<0.05 vs EGF alone; $^{!!}p$<0.01 vs a combination of bFGF and EGF, Tukey's test following ANOVA). Data represent means±s.e.m. of 4 cultures.

activate both Ras/MAPK and PI3K pathways in order to modulate GLAST expression.

Although S6 kinase (Volarevic and Thomas, 2000) and phospholipase C (PLC) (Kamat and Carpenter, 1997) have also been identified as downstream signaling molecules of growth factor receptors, neither the inhibitor of S6 kinase rapamycin nor the inhibitor of PLC U73122 blocked the effect of the growth factors.

Intracellular Ca^{2+} is known as a second messenger of gene regulation and thereby induce diverse transcriptional responses (Bading et al., 1997). The possible contribution of Ca^{2+} to the GLAST regulation was assessed by examining the effect of the intracellular Ca^{2+} chelator BAPTA-AM. BAPTA-AM abolished the effect of bFGF, but the effect of IGF-1 or EGF were substantially unaffected. The $[Ca^{2+}]_i$ elevation may be required only for the bFGF receptor signaling. Therefore, we investigated the involvement of protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), both of which are involved in Ca^{2+}-activated signaling pathway. Unexpectedly, the inhibitor of PKC calphostin C had no influence on the effect of bFGF but rather blocked the effect of IGF-1 and EGF. The inhibitor of CaMKII KN93 had no effect on growth factor-increased glutamate uptake.

Table 1. Effect of inhibitors of various signaling cascades on growth factor-enhanced glutamate uptake activities

Drugs	Control (% of baseline uptake activity)	bFGF (% of the effect of each growth factor)	IGF-1 (% of the effect of each growth factor)	EGF (% of the effect of each growth factor)
–	100.0	100.0	100.0	100.0
Herbimycin A	97.2±8.5	10.8±5.6‡	24.9±5.4‡	24.0±17.7‡
Genistein	97.7±4.2	10.0±12.2‡	86.8±12.6	–6.1±8.6‡
AFC	95.4±7.7	–0.2±2.1‡	22.8±8.3‡	21.5±4.8‡
PD98059	93.3±3.5	–9.2±7.0‡	49.1±8.3*	33.0±11.8*
Wortmannin	97.6±2.53	2.6±7.1*	56.9±5.9*	38.6±15.2*
Rapamycin	100.4±7.7	86.4±7.6	80.1±10.9	83.9±21.0
U73122	107.9±3.5	82.3±20.6	79.2±3.9	114.1±21.1
BAPTA-AM	104.8±2.7	52.1±2.5*	96.5±1.5	124.8±15.0
Calphostin C	94.6±0.97	5.7±20.7	32.9±4.8‡	41.9±23.7*
KN-93	107.1±5.3	124.7±12.5	90.3±6.9	123.2±13.5

In the control experiment, astrocytes were treated with various inhibitors for 36 hours, and then glutamate uptake activity was measured. Data are expressed as a percentage of untreated astrocytes. In the other experiments, astrocytes were treated for 36 hours with either 3 ng/ml bFGF, 100 ng/ml IGF-1 or 30 ng/ml EGF in the presence of various inhibitors. Data are expressed as a ratio to the growth factor-facilitated uptake, which are determined by (values in the inhibitor-cotreated groups – the baseline values) / (values in the growth factor-treated groups – the baseline values) × 100. The concentration of each drug is as follows: 10 μM herbimycin A, 10 μM genistein, 20 μM AFC, 30 μM PD98059, 1 μM wortmannin, 1 μM rapamycin, 10 μM U73122, 5 μM BAPTA-AM, 100 nM calphostin C and 10 μM KN-93.

*p<0.05.

‡p<0.01 vs the corresponding growth factor, Tukey's test following ANOVA). Data represent means±s.e.m. of 4 or 5 cultures.

Incidentally, none of these inhibitors used in this study affected the baseline glutamate uptake activity.

DISCUSSION

Growth factors were initially identified as potent mitogenic peptides, and are currently known as multifunctional molecules in diverse types of cells. In spite of widespread distribution of growth factors and their receptors in the developing and mature central nervous system, the precise physiological function of these growth factors in the brain has not yet been fully determined. Here we have shown for the first time that expression of the glial Na^+-dependent glutamate transporter GLAST in cortical astrocytes is regulated by bFGF, IGF-1 and EGF in a time- and concentration-dependent manner. However, it is unlikely that all known growth factors equally exert the same effect, because insulin, PDGF-AB and HGF was ineffective in the present study. The receptor for insulin (Zhu et al., 1990), PDGF-AB (Zhang and Hutchins, 1996) and HGF (Welch et al., 1999) are expressed in astrocytes. Therefore, the lack of an effect is possibly explained by the ability of the corresponding receptors to activate a specific signaling pathway for GLAST regulation.

In the primary cultures, astrocytes show a polygonal morphology and express only GLAST. Expression of the other astrocytic transporter GLT-1 is generally thought to depend on neuron-derived factors (Swanson et al., 1997). Indeed, Zelenaia et al. reported that long-term treatment with EGF, an assumed neuronal signal, can induce the GLT-1 expression in immature astrocytes (Zelenaia et al., 2000). However, the neuronal signal is not unique to the GLT-1 regulation. The observation that destruction of glutamatergic corticostriatal projections causes the downregulation of GLAST as well as GLT-1 (Levy et al., 1995) suggests that normal astroglial/neuron interactions are also important for the GLAST regulation. However, molecular mechanisms underlying the GLAST regulation have not been elucidated. In the present study, bFGF, IGF-1 and EGF induced an increase in GLAST expression levels. GLT-1 expression was unchanged by these growth factors for at least 0.5 to 48 hours of exposure.

Despite the continuous presence of the growth factors, the GLAST upregulation was a transient response. Growth factor-mediated increases in glutamate uptake activity were well accompanied by changes in GLAST mRNA levels, which suggests that the growth factors modulate the transcriptional level. Indeed, the slightly preceding increase in GLAST mRNA may reflect a translational process. Another possibility is that the increased GLAST mRNA is due to reduced mRNA degradation. In any case, however, the transient regulation by growth factors may be a key property that may allow a dynamic and delicate control of GLAST expression in astrocytes. In contrast with the previous report (Zelenaia et al., 2000), we found no evidence that EGF induced GLT-1 expression. This inconsistency may be attributable to the duration of the EGF treatment. In their experiments, EGF was applied for 7 days. The GLT-1 induction may be required for such prolonged EGF stimulation.

In some experimental models, GLAST appears to exhibit higher K_m values than those of GLT-1, and is thus suspected of predominantly operating at pathologically elevated concentrations of glutamate (Kanai et al., 1997). It has already been reported that growth factors such as bFGF, IGF-1 and EGF prevent neurons from glutamate-mediated excitotoxicity (Abe and Saito, 1992; Maiese et al., 1993; Freese et al., 1992; Skaper et al., 1993; Nakao et al., 1996). Therefore, the protective effects of the growth factors are of interest with respect to glutamate transporter regulation. In light of our findings, the growth factors may facilitate clearance of glutamate under pathological conditions via GLAST upregulation. The observation that expression of bFGF increases following brain injury in vivo (Logan et al., 1992; Reilly and Kumari, 1996) raises the possibility that such protective mechanisms actually operate in the brain.

bFGF is known to bind to heparan sulfate proteoglycans at the cell surface and to the high-affinity FGF receptor, which contains a cytoplasmic tyrosine kinase domain (Givol and Yayon, 1992). bFGF receptor activation causes an increase in tyrosine kinase activity, and triggers phosphorylation of several proteins including the receptor itself, which is believed to be the initial step leading to biological actions of bFGF (Givol and Yayon, 1992). Similarly, IGF-1 and EGF bind and activate their receptor tyrosine kinases (Leof, 2000). Therefore it is possible that these growth factors stimulate a common signaling pathway to regulate GLAST expression. Indeed, the effects of all three growth factors were inhibited by herbimycin A, AFC, PD98059 or wortmannin. These results suggest that all these factors regulate GLAST expression via Ras/MAK and PI3K signaling cascades. However, these growth factors are unlikely to completely share the same signal transduction pathway.

A combination of IGF-1 with either bFGF or EGF resulted in a further increase in glutamate uptake activity, which strongly suggests that IGF-1 activates a different signaling pathway. This notion is further supported by a pharmacological study showing that genistein inhibited the effect of bFGF and EGF without affecting the effect of IGF-1. Genistein-sensitive and insensitive pathways may independently mediate the growth factor-induced regulation of GLAST expression. Interestingly, the effects of the growth factors were equally blocked by herbimycin A, a selective inhibitor of Src family kinases (Uehara et al., 1989), whereas genistein is known to be a relatively low selective inhibitor of protein tyrosine kinase (Akiyama et al., 1987; Uehara, 1997). Therefore, the tyrosine kinase involved specifically in IGF-1-activated signaling pathway cannot be deduced from our present data. A combination of bFGF and EGF had no additive effect on glutamate uptake activity, which suggests that bFGF and EGF may, at least in part, activate a common pathway. However, the effect of bFGF requires a $[Ca^{2+}]_i$ rise, whereas the effect of EGF is dependent on PKC activation, but not vice versa. bFGF and EGF may use independent signaling pathways initially but converge at some later step in the induction of GLAST.

In conclusion, bFGF, IGF-1, and EGF modulate the expression of the glial Na^+-dependent glutamate transporter through heterogenous signaling pathways. Currently, little is known about the promoter region of GLAST gene. Promoter analysis may elucidate these complicated regulations of GLAST by the growth factors. To our knowledge, our finding is the first evidence that glutamate transporters receive temporally tight regulation of gene expression (i.e. transient upregulation). The present study may provide a novel regulatory mechanism of glutamate transporters, and thus may

be useful in exploring a new approach for preventing neurological diseases associated with glutamatergic neurotoxicity.

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