Platelet-derived Growth Factor Rapidly Increases Activity and Cell Surface Expression of the EAAC1 Subtype of Glutamate Transporter through Activation of Phosphatidylinositol 3-Kinase*

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Na⁺-dependent glutamate transporters are the primary mechanism for removal of excitatory amino acids (EAs) from the extracellular space of the central nervous system and influence both physiologic and pathologic effects of these compounds. Recent evidence suggests that the activity and cell surface expression of a neuronal subtype of glutamate transporter, EAAC1, are rapidly increased by direct activation of protein kinase C and are decreased by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3-K). We hypothesized that this regulation could be analogous to insulin-induced stimulation of the GLUT4 subtype of glucose transporter, which is dependent upon activation of PI3-K. Using C6 glioma, a cell line that endogenously expresses the neuronal subtype of glutamate transporter, which is dependent upon activation of PI3-K. Using C6 glioma, a cell line that endogenously expresses EAAC1, we report that platelet-derived growth factor (PDGF) increased Na⁺-dependent [³H]glutamate transport activity within 30 min. This effect of PDGF was not due to a change in total cellular EAAC1 immunoreactivity but was instead correlated with an increase in cell surface expression of EAAC1, as measured using a membrane impermeant biotinylation reagent combined with Western blotting. A decrease in nonbiotinylated intracellular EAAC1 was also observed. These studies suggest that PDGF causes a redistribution of EAAC1 from an intracellular compartment to the cell surface. These effects of PDGF were accompanied by a 35-fold increase in PI3-K activity and were blocked by the PI3-K inhibitors, wortmannin and LY 294002, but not by an inhibitor of protein kinase C. Other growth factors, including insulin, nerve growth factor, and epidermal growth factor, had no effect on glutamate transport nor did they increase PI3-K activity. These studies suggest that, as is observed for insulin-mediated translocation of GLUT4, EAAC1 cell surface expression can be rapidly increased by PDGF through activation of PI3-K. It is possible that this PDGF-mediated increase in EAAC1 activity may contribute to the previously demonstrated neuroprotective effects of PDGF.

The rapid clearance of glutamate from the extracellular space of the central nervous system by Na⁺-dependent high affinity glutamate transporters is critical to the maintenance of effective synaptic transmission and the prevention of excitotoxic injury. Increases in extracellular EAs¹ after head trauma and ischemic events have been described (1–3) and are presumably related to both a failure of inward transport and increased reverse operation of the carriers (4, 5). A family of glutamate transporters mediates this high affinity uptake and includes five members, the glial transporters GLT-1 (human homologue EAAT2) and GLAST (EAAT1), the neuronal transporters EAAC1 (EAAT3) and EAAT4, and the retinal transporter EAAT5 (6–10). The EAAC1 subtype of transporter is enriched in the pyramidal cells of hippocampus and cortex (11, 12), two areas rich in glutamatergic transmission and exquisitely sensitive to excitotoxic insults (13). Animals treated with antisense oligonucleotides to “knock down” EAAC1 expression develop a seizure phenotype, suggesting a role for EAAC1 in dampening excitability (14).

The EAAC1 subtype is also expressed in several peripheral tissues including the kidney and intestine (11, 15). Although some results suggest mRNAs for the other transporters are expressed in selected peripheral tissues (for reviews see Refs. 16 and 17), two studies have not observed protein expression (11, 18). These studies suggest that EAAC1 may uniquely regulate extracellular acidic amino acids in the periphery. In fact, mice genetically deleted of EAAC1 excrete abnormally high levels of acidic amino acids in the urine, suggesting that this transporter mediates reabsorption of glutamate and aspartate from the glomerular filtrate (19). Therefore, understanding the acute regulation of this transporter may help elucidate its function during excitatory transmission and excitotoxic events, as well as in peripheral glutamate metabolism.

Recent studies demonstrate that the activity of several neurotransmitter transporters can be rapidly regulated by direct activation of intracellular signaling molecules (PKC or cAMP-dependent protein kinase), including norepinephrine (20), serotonin (21), dopamine (22–24), GABA (25, 26), and glutamate transporters (27). In many cases, the changes in activity are correlated with a redistribution of transporter protein from the cell surface to an intracellular compartment or vice versa. There is also evidence that some of these transporters are regulated by their substrates. For example, Bernstein and Quick (28) recently demonstrated that GABA and other GABA transporter substrates increase the activity and cell surface

¹ The abbreviations used are: EAA, excitatory amino acid; PKC, protein kinase C; GABA, γ-aminobutyric acid; PI3-K, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; DMEM, Dulbecco’s modified Eagle medium; BSA, bovine serum albumin; NGF, nerve growth factor; EGF, epidermal growth factor; PLC-γ, phospholipase C-γ; MAP kinase, mitogen-activated protein kinase; Bis II, bisindolylmaleimide II; PKB, protein kinase B.
expression of the GAT1 subtype of GABA transporter. Ramamoorthy and Blakely (29) provide evidence that serotonin transporter substrates decrease PKC-dependent phosphorylation and internalization of the serotonin transporter. Little is known about receptor-mediated regulation of transporter function, but there is evidence that histamine and adenosine receptor activation regulate serotonin transporter function by an unknown mechanism (30, 31). Recent studies have demonstrated that activation of G protein-coupled receptors causes a decrease in cell surface expression of the GAT1 subtype of GABA transporter in neurons (32). Angiotensin II and insulin may regulate norepinephrine transport in spontaneously hypertensive rats (33) and SK-N-SH cells (34), respectively. Both of these effects appear to be dependent on PI3-K.

We recently demonstrated that activation of PKC with phorbol ester increases the activity and cell surface expression of the EAAC1 subtype of glutamate transporter (27). Except for a PKC-induced increase in GAT1 cell surface expression observed in Xenopus oocytes (25), this increase in EAAC1 cell surface expression following PKC activation is unique. We also found that wortmannin, an inhibitor of PI3-K, decreased EAAC1 activity and cell surface expression. These regulated changes in activity and cell surface expression qualitatively resemble translocation events observed for the insulin-sensitive glucose transporter GLUT4 (reviewed in Refs. 35 and 36). The regulation of GLUT4 has been well studied and appears to be primarily mediated by activation of the insulin receptor tyrosine kinase cascade and stimulation of PI3-K, although some studies suggest an additional role for phorbol 12-myristate 13-acetate (PMA)-sensitive PKCs.

In the present study, the effects of growth factors on the activity of EAAC1 were examined using C6 glioma as a model system that selectively and endogenously expresses this subtype of transporter. Of the growth factors tested, only PDGF stimulated PI3-K activity and rapidly (within minutes) increased both the activity and cell surface expression of EAAC1. These three effects of PDGF were blocked by two different inhibitors of PI3-K. Although the effects of PDGF and phorbol ester on activity and cell surface expression of EAAC1 were not additive, a PKC inhibitor did not block the effects of PDGF. These studies strongly suggest that EAAC1 cell surface expression and activity can be regulated within minutes by two independent but converging signaling pathways. This regulation may provide a novel mechanism to limit extracellular glutamate accumulation in the central nervous system.

**EXPERIMENTAL PROCEDURES**

**Materials—**DMEM, L-glutamine, and penicillin/streptomycin were purchased from Life Technologies. Fetal bovine serum was from HyClone (Logan, UT). Twelve-well and 10-cm tissue culture plates were manufactured by Corning Costar (Corning, NY). All radioisotopes were obtained from Amersham Pharmacia Biotech. and polyvinylidene fluoride Immobilon P membranes were from Millipore (Bedford, MA). Lipids for the PI3-K assay were from Avanti (Alabaster, AL). Anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Silica gel-coated thin layer chromatography plates were from Merck.

**Cell Culture**—The rat central nervous system-derived cell line C6 glioma was used in the present study endogenously and exclusively expresses the EAAC1 subtype of transporter (27, 37, 38) and makes an ideal model system to examine the regulation of EAAC1 in isolation. C6 glioma cells were obtained from American Type Culture Collection.
reaction mixture followed by 10 μl of phosphatidylinositol substrate (2 mg/ml sonicated in 10 mM Tris HCl (pH 7.5) + 1 mM EGTA). The kinase reaction was started by adding 10 μl of 440 μM ATP containing 30 μCi [γ-32P]-ATP to each tube, and tubes were incubated for 10 min at room temperature. The reaction was halted by the addition of 20 μl 8 M HCl and 160 μl chloroform:methanol (1:1). The tubes were then centrifuged and the organic phase was removed. 20 μl of the organic phase was spotted on a silica gel-coated thin-layer chromatography plate impregnated with 1% potassium oxalate. Phospholipids were resolved in chloroform:methanol:water:ammonium hydroxide (60:47:11:3:2). Radioactivity that co-migrated with an authentic phosphatidylinositol 4-phosphate standard was measured using a PhosphoImager SI (Molecular Dynamics, Sunnyvale, CA).

### Assessment of Cell Surface Expression—Biotinylation of Cell Surface Proteins

In C6 glioma, the EAAC1 transporter at each concentration of D-[3H]aspartate (*, p < 0.05).
PDGF Regulates EAAC1 Cell Surface Expression via PI3-K

PDGF is biologically active as a dimer consisting of either hetero- or homodimers of A or B chains, whereas PDGF receptors are composed of either α or β subunits (for review see Ref. 52). Ligands containing the B chain can activate either receptor subunit, but the PDGF A chain only activates receptors containing the α subunit. To determine if activation of the α subunit is sufficient to explain the observed increase in transport activity, the effects of a maximal concentration of PDGF AA (20 ng/ml) on transport activity were examined. At this concentration, PDGF AA had no significant effect on transport activity (121 ± 12% of control; n = 5), compared with an increase to 151 ± 9% of control by PDGF BB in parallel experiments (PDGF BB versus Control, p < 0.01; PDGF BB versus PDGF AA, p < 0.05; n = 5). This suggests that activation of the α receptor subtype may not contribute to the effects of PDGF BB on glutamate transport in C6 glioma, or the α subunit may not be expressed in this cell line. To study the maximal effects of PDGF BB stimulation, a concentration of 20 ng/ml was used in all subsequent experiments. This concentration of PDGF BB approximates that used by others to activate PDGF receptor-dependent signaling pathways (53, 54).

The mechanism of PDGF stimulation of Na⁺-dependent glutamate uptake was assessed by determining if uptake capacity or substrate affinity changed. PDGF BB (20 ng/ml) increased uptake activity by nearly doubling the V_{max} of EAAC1-mediated transport from 660 ± 60 pmol/mg/min to 1180 ± 80 pmol/mg/min (p < 0.001, see Fig. 2A). The K_{m} of transport was also slightly increased from 12.2 ± 0.7 μM to 15.4 ± 0.9 μM (p < 0.05), but the effect is probably not physiologically relevant compared with the large change in V_{max} observed. To rule out the possibility that PDGF artifactually increased transport activity by increasing intracellular glutamate metabolism, we tested the effects of PDGF treatment on EAAC1-mediated uptake of the nonmetabolizable EAAC1 substrate, D-[³H]aspartate. At three concentrations of D-aspartate that ranged from approximately 10-fold below to approximately 10-fold above its K_{m} value (27), PDGF caused a significant increase in Na⁺-dependent PDGF, NGF, EGF, insulin, or vehicle for 30 min prior to immunoprecipitation of PI3-K with an anti-phosphotyrosine antibody. Data are the mean ± S.E. of at least three independent experiments and are expressed as a percentage of control PI3-K activity levels. PDGF increased PI3-K activity to 4500 ± 700% of control (p < 0.001), whereas the other growth factors had no significant effects (NGF, 88 ± 10% of control; EGF, 113 ± 19% of control; insulin, 194 ± 64% of control; n = 3–5). C, effects of PDGF and PI3-K inhibitors on the activity of PI3-K. Cells were treated with PDGF (20 ng/ml) or vehicle for 30 min prior to the start of the PI3-K assay. Prior to the addition of substrates, either 100 μM wortmannin or 100 μM LY 294002 was added as indicated. Data are presented as percentage of control PI3-K activity and are means ± S.E. of at least three independent experiments. PDGF increases PI3-K activity by approximately 35-fold (*, p < 0.001). Wortmannin and LY 294002 alone decrease basal PI3-K activity to undetectable levels and fully block the stimulation of PDGF (to 42 ± 21% and 110 ± 29% of control, respectively).

![Fig. 3. The effects of PDGF and PI3-K inhibitors on EAAC1 uptake activity and PI3-K signaling.](http://www.jbc.org/)

A. Effects of a PDGF receptor tyrosine kinase inhibitor and PI3-K inhibitors on PDGF stimulation of Na⁺-dependent L-[³H]glutamate uptake in C6 glioma. Cells were pre-treated for 5 min with either vehicle (MeSO₄), 50 μM tyrphostin AG1295, 100 μM wortmannin, or 100 μM LY 294002 followed by the addition of 20 ng/ml PDGF or vehicle (4 mM HCl containing 0.01% BSA) for 30 min prior to assessment of Na⁺-dependent L-[³H]glutamate uptake. Data are presented as the mean ± S.E. of four to eight independent experiments. PDGF significantly increased uptake, whereas wortmannin significantly decreased uptake of L-[³H]glutamate (Control versus PDGF and Control versus wortmannin, *, p < 0.001). LY294004 and tyrphostin AG1295 had no effect on transport. All inhibitors blocked the effects of PDGF on Na⁺-dependent L-[³H]glutamate uptake (no statistical differences from control, PDGF versus wortmannin plus PDGF and PDGF versus LY 294002 plus PDGF, ***, p < 0.05). B. Effects of growth factors on PI3-K activity. Cells were treated with 20 ng/ml PDGF, NGF, EGF, insulin, or vehicle for 30 min prior to immunoprecipitation of PI3-K with an anti-phosphotyrosine antibody. Data are the mean ± S.E. of at least three independent experiments and are expressed as percentage of control PI3-K activity levels. PDGF increased PI3-K activity to 4500 ± 700% of control (p < 0.001), whereas the other growth factors had no significant effects (NGF, 88 ± 10% of control; EGF, 113 ± 19% of control; insulin, 194 ± 64% of control; n = 3–5). C, effects of PDGF and PI3-K inhibitors on the activity of PI3-K. Cells were treated with PDGF (20 ng/ml) or vehicle for 30 min prior to the start of the PI3-K assay. Prior to the addition of substrates, either 100 μM wortmannin or 100 μM LY 294002 was added as indicated. Data are presented as percentage of control PI3-K activity and are means ± S.E. of at least three independent experiments. PDGF increases PI3-K activity by approximately 35-fold (*, p < 0.001). Wortmannin and LY 294002 alone decrease basal PI3-K activity to undetectable levels and fully block the stimulation of PDGF (to 42 ± 21% and 110 ± 29% of control, respectively).
dependent d-aspartate transport (Fig. 2B). The percentage increase was comparable at all three concentrations, consistent with an increase in $V_{\text{max}}$. To determine if PDGF increases transport activity by nonspecifically altering the electrochemical gradients of the cell, the effects of PDGF on Na$^+$-dependent transport of $[^3\text{H}]$glycine were also examined. PDGF caused no significant changes in Na$^+$-dependent $[^3\text{H}]$glycine uptake at the two lower concentrations of glycine, providing indirect evidence that the increase in glutamate transport activity cannot be attributed to an alteration in the Na$^+$ electrochemical gradient of the cell (Fig. 2C). There was a significant increase in PDGF-stimulated glycine uptake at the highest concentration of glycine, but this increase was much smaller than the near doubling observed for both glutamate and aspartate transport. This suggests that a low affinity glycine transport system may be slightly enhanced by PDGF treatment.

Identification of Signaling Pathways Involved in the Stimulation of EAAC1-mediated Glutamate Uptake by PDGF—After ligand-induced receptor dimerization and activation of an intrinsic tyrosine kinase, the PDGF receptor can activate several signaling pathways, including PI3-K, phospholipase C-γ (PLC-γ), and mitogen-activated protein kinase (MAP kinase). To examine the specificity of the effects of PDGF, a selective inhibitor of the PDGF receptor tyrosine kinase, tyrphostin AG1295, was used to determine if the changes in EAAC1-mediated glutamate uptake were dependent on activation of the PDGF receptor tyrosine kinase. Although tyrphostin AG1295 alone had no effect on transport activity, it completely abolished the effects of PDGF (Fig. 3A).

To determine if the actions of PDGF on glutamate uptake are dependent on PI3-K activation, the effects of two different inhibitors of PI3-K (wortmannin and LY 294002) were examined. Both inhibitors were used at concentrations that are approximately 10- to 20-fold above their published IC$_{50}$ values (55, 56). At these concentrations, treatment of C6 glioma with wortmannin reduced transport activity below control levels as was previously observed (Fig. 3A) (27). This effect was specific for wortmannin as LY 294002 alone had no effect on basal transport activity. Both inhibitors significantly attenuated the PDGF-induced increases in transport activity (Fig. 3A).

PDGF, EGF, NGF, and insulin can all activate PI3-K in other cell types (for reviews see Refs. 57 and 58), but their functional coupling to PI3-K has not been examined in C6 glioma. Therefore, the effects of these growth factors on PI3-K activity were examined. In the present study, only PDGF caused a detectable increase in PI3-K activity (Fig. 3B); $^{32}$P-labeling of phosphatidylinositol increased to 3500 ± 700% of control ($n = 7$, $p < 0.001$). This demonstrates functional coupling of PDGF receptors to PI3-K in C6 glioma. In addition, both wortmannin and LY 294002 blocked basal PI3-K activity and significantly attenuated PDGF stimulation of PI3-K at the same concentrations that inhibited the effects of PDGF on EAAC1-mediated glutamate transport activity (Fig. 3C). These studies suggest that the effects of PDGF on Na$^+$-dependent glutamate transport activity are dependent on activation of PI3-K.

Effects of PDGF and PI3-K Inhibition on Cell Surface Expression of EAAC1—To define the mechanism of this PDGF-dependent increase in transport activity, a membrane impermeant biotinylation reagent was used to modify cell surface proteins, allowing batch extraction and subsequent Western blot analysis to measure the fraction of transporters on the cell surface after treatment with PDGF. These Western blots were probed with an anti-actin antibody as a control for possible nonspecific effects of a treatment on membrane permeability. Under control conditions, the percentage of biotinylated EAAC1 cell surface expression via PI3-K  

| Parameter          | Mean ± S.E. |
|--------------------|-------------|
| Control cells      |             |
| % Biotinylated actin | 13 ± 3%    |
| % Biotinylated EAAC1 | 45 ± 3%    |
| PDGF-treated cells |             |
| Biotinylated actin | 115 ± 22 (% of control, NS) |
| Biotinylated EAAC1 | 168 ± 14 (% of control)*   |
| Nonbiotinylated EAAC1 | 81 ± 7 (% of control)*   |

EAAC1 immunoreactivity was 45 ± 3%, and the percentage of biotinylated actin was 13 ± 3% across all experiments (Table I, see Figs. 4 and 5 for examples). The small amount of biotinylated actin is presumably related to cell lysis and suggests that this procedure may slightly overestimate the proportion of transporter that resides on the cell surface under baseline conditions. Consistent with the time course observed for PDGF stimulation of glutamate uptake, no changes in the total amount of EAAC1 immunoreactivity were observed following any of the treatments, suggesting no net synthesis or degradation of transporters (see Figs. 4 and 5, B and C; total cell lysate). PDGF increased EAAC1 immunoreactivity to 168 ± 14% ($p < 0.001$) in the biotinylated or cell surface fraction across all experiments and significantly decreased the amount of nonbiotinylated intracellular transporter to 81 ± 7% of control ($p < 0.05$; see Table I). This increase in the cell surface expression of transporter correlates with the increase in glutamate uptake activity observed after PDGF treatment. Importantly, PDGF had no effect on the amount of biotinylated actin (Table I), suggesting that this increase in biotinylated EAAC1 immunoreactivity cannot be attributed to a nonspecific increase in membrane permeability but is instead caused by an increase in the number of transporters at the cell surface.

To determine if the PDGF-mediated increases in cell surface expression of EAAC1 are dependent on activation of PI3-K, the effects of both wortmannin and LY 294002 treatment were examined. Although LY 294002 alone had no effect on cell surface expression (Fig. 4D), wortmannin decreased cell surface expression levels below those observed in vehicle-treated cells, paralleling its effects on uptake activity (Fig. 4B). Both inhibitors of PI3-K completely attenuated the PDGF-mediated increases in EAAC1 cell surface expression (Fig. 4). This suggests, as observed for the increase in transport activity, that the effects of PDGF on EAAC1 cell surface expression are mediated through activation of PI3-K.

Effects of Inhibition of Other Targets of the PDGF Receptor Tyrosine Kinase—The PDGF receptor can also activate other downstream signaling targets including PLC-γ and MAP kinase (for review see Ref. 52). A major product of PLC-γ, diacylglycerol, is an activator of classical PKCs. Therefore, it is possible that the effects of PDGF are dependent on activation of the same phorbol ester-activated PKC that has previously been shown to regulate EAAC1 (27). To examine this possibility, C6 glioma were treated with PDGF, PMA, or the PKC inhibitor bisindolylmaleimide II (Bis II). The concentration of Bis II (10 μM) used in this study has been previously shown to completely inhibit PMA-induced increases in EAAC1-mediated glutamate uptake. 

Summary of effects of PDGF on biotinylated and nonbiotinylated actin and EAAC1 immunoreactivity

C6 glioma were treated with either vehicle (Control) or PDGF (20 ng/ml) prior to biotinylation with a membrane impermeant biotin reagent. After cell lysis, biotinylated proteins were batch extracted, and total cell lysate, nonbiotinylated (intracellular) and biotinylated (cell surface) fractions were analyzed by Western blot. Relative changes in EAAC1 and actin immunoreactivity were assessed by quantitation of films using NIH Image software. Data are presented as the mean ± S.E. of 15 independent experiments ($^* p < 0.05; ^{**} p < 0.001$ compared to control values; NS, not significant). The percent yield of transporter was 82 ± 7% across all experiments.
uptake and cell surface expression (27). As shown in Fig. 5A, PDGF increased glutamate uptake to approximately 150% of control, whereas PMA increased activity to nearly 200% of control. Bis II treatment alone had no effect on transport and did not inhibit the PDGF-stimulated increase in transport activity, but completely blocked the effects of PMA (Fig. 5A). To determine if PKC is involved in the PDGF-induced increase in EAAC1 cell surface expression, cells were preincubated with Bis II prior to treatment with PDGF, and cell surface expression was evaluated using biotinylation. Bis II had no effect on the PDGF-mediated increase in cell surface expression (Fig. 5, B and C), but in experiments performed concurrently with the PDGF studies Bis II completely blocked the PMA-induced increase in biotinylated EAAC1 (PMA alone, 221 ± 41%; PMA plus Bis II, 88 ± 16% of control; n = 3, p < 0.01; data not shown). These data suggest that the PDGF-induced stimulation of EAAC1 cell surface expression is not regulated by a phorbol ester- or PLC-γ-activated PKC isoform, because most of these isoforms are inhibited by Bis II at the concentrations used (59). These studies do not rule out the involvement of an atypical or novel PKC isoform that is phorbol ester/diacylglycerol insensitive, because several have been identified as downstream effectors of PI3-K (for review see Ref. 60). However, based on their reported sensitivity to bisindolylmaleimides (IC50 values in the micromolar range, see Ref. 59), one would expect that the concentrations used in the present study would at least partially block these atypical or novel isoforms.

Although these studies suggest that the effects of PDGF are not dependent on a Bis II-sensitive PKC pathway, it is possible that the effects of PDGF and PMA converge on a common signaling pathway. To address this possibility, cells were incubated with PMA and PDGF simultaneously to determine if...
their effects are additive. Using concentrations that produced maximal stimulation of transport activity, PDGF (20 ng/ml) increased activity to 170 ± 628%, PMA (100 nM) increased activity to 222 ± 36%, and co-application of PDGF and PMA increased activity to 203 ± 20%, not significantly different from either PMA alone or PDGF alone (n = 4, data not shown).

Similarly, preliminary biotinylation experiments show no additive effect of PMA and PDGF on cell surface expression (PDGF increased cell surface expression to 148% of control, PMA to 197% of control, and PDGF plus PMA to 213% of control; n = 2, data not shown). This suggests that the effects of PMA may converge with PDGF either upstream or downstream of PI3-K. Although we found no precedent for PKC-mediated activation of PI3-K, the effects of PMA on PI3-K activity were examined to determine if PMA activates this kinase in C6 glioma. PMA caused no increase in PI3-K immunoprecipitated with the same anti-phosphotyrosine antibody used to demonstrate PDGF-mediated stimulation of PI3-K (labeled PIP was 85 ± 6% of control, n = 4, data not shown), but we cannot rule out the possibility that a different PI3-K isoform may be activated. This suggests that the effects of PMA and PDGF converge at a signaling molecule downstream of PI3-K, that they both regulate the same step in transporter redistribution, or that they regulate the same intracellular pool of transporter.

The PDGF receptor tyrosine kinase cascade has additional targets including MAP kinase and may proceed through sig-

**Fig. 5.** Effects of the PKC inhibitor Bis II on PDGF- and PMA-stimulated L-[3H]-glutamate uptake activity and cell surface expression. A, the effects of PDGF, PMA, and a PKC inhibitor on glutamate uptake activity. Cells were pretreated for 5 min with either 10 μM Bis II or vehicle (MeSO) followed by the addition of 20 ng/ml PDGF, 100 nM PMA, or vehicle (MeSO) for 30 min prior to measurement of Na+-dependent L-[3H]glutamate uptake. Data are presented as the mean ± S.E. of three to eight independent experiments performed in triplicate. PDGF and PMA significantly increased L-[3H]glutamate uptake (Control versus PDGF and Control versus PMA; *, p < 0.001). Bis II pretreatment had no effect on PDGF stimulation but fully blocked PMA stimulation of L-[3H]glutamate uptake (Control versus Bis II plus PDGF; *, p < 0.001; PMA plus Bis II not significantly different from control). B, representative immunoblot of Bis II effects on EAAC1 (66 and 220 kDa and larger bands) and actin (46 kDa) immunoreactivity in the total cell lysate, intracellular, and biotinylated (cell surface) fractions. C6 glioma were pretreated for 5 min with either 10 μM Bis II or vehicle (MeSO) followed by the addition of 20 ng/ml PDGF or vehicle (4 mM HCl containing 0.01% BSA) for 30 min prior to biotinylation. C, quantitation of EAAC1 immunoreactivity from five independent experiments (mean ± S.E.) demonstrating an increase in biotinylated EAAC1 following PDGF treatment. Bis II alone had no effect on biotinylated EAAC1 and had no effect on the PDGF-induced stimulation of cell surface EAAC1 (Control versus PDGF, Control versus PDGF plus Bis II; *, p < 0.05). No significant changes in total cell lysate or intracellular EAAC1 were observed with any treatments. In three of the five experiments, PMA was used in parallel to increase cell surface expression of EAAC1, and Bis II completely blocked these PMA-induced increases (data not shown, see "Results").
PDGF Regulates EAAC1 Cell Surface Expression via PI3-K

The ability of PDGF to increase EAAC1 transport activity and cell surface expression in a PI3-K-dependent fashion qualitatively resembles the regulation of GLUT4 in the periphery. In 3T3-L1 adipocytes and several other model systems, insulin increases activity and cell surface expression of the GLUT4 subtype of glucose transporter (reviewed in Refs. 35 and 36). As is observed for the effects of PDGF on EAAC1, these effects of insulin occur within minutes and are blocked by both wortmannin and LY 294002 (41, 42). Although some earlier studies had not observed effects of PDGF on glucose transport, Wang et al. (70) recently reported that PDGF rapidly induces a 6-fold increase in glucose transport using 3T3-L1 adipocytes as a model system (for a discussion of earlier literature, see Ref. 70). This increase in activity was correlated with an increase in cell surface expression of a myc-tagged GLUT4 transporter. As was observed in the present study, these effects of PDGF were accompanied by an increase in PI3-K activity and were blocked by wortmannin (100 nM), suggesting that the effects of PDGF on GLUT4 and EAAC1 may be comparable. Although qualitatively similar, a larger percentage of GLUT4 appears to be sequestered intracellularly, and insulin has a greater effect on glucose uptake activity and translocation (up to 20-fold) than is observed after PDGF stimulation of EAAC1 (reviewed in Refs. 36 and 71). Although it is possible that this quantitative difference in intracellular sequestration is related to differences intrinsic to EAAC1 and GLUT4, it is also possible that under different conditions EAAC1 is more effectively retained intracellularly. It has also been suggested that GLUT4 transporters are segregated into two intracellular compartments, a constitutively recycling pool and a rapidly regulated pool of transporters (72–74) (reviewed in Ref. 36). One argument for two compartments is the observation that transferrin receptors, a marker of the endosomal recycling compartment, and GLUT4 do not always co-localize (73, 75) (for review see Ref. 71). In fact, a substantial portion of cytoplasmic EAAC1 does not co-localize with transferrin receptors in control and wortmannin-treated C6 glioma, suggesting that intracellular EAAC1 may also segregate to two distinct intracellular compartments. Together, these observations suggest many similarities between the regulated trafficking of EAAC1 and GLUT4.

The two most common PI3-K inhibitors, wortmannin and LY 294002, both blocked the effects of PDGF on EAAC1 uptake activity and cell surface expression, but in the absence of PDGF these two compounds had different effects. Wortmannin decreased basal glutamate uptake and cell surface localization of EAAC1, allowing examination of the transporter in isolation. C6 glioma are an undifferentiated cell line of central nervous system origin that express both neuronal (glutamic acid decarboxylase) and glial (glutamine synthetase and glial fibrillary acidic protein) markers (63–65). Although neurons in culture might more closely mimic the cellular milieu observed in vivo, we find that four transporters (EAAC1, EAAT4, GLUT1, and GLAST) are expressed in these cultures. This expression of additional transporters may be due to glial contamination, as well as changes in transporter expression properties of neurons grown in culture (66, 67). Additionally, the pharmacology of transport activity in these cultures suggests that at least three of these transporters contribute to glutamate uptake activity (68). Because there are no specific inhibitors of EAAC1-mediated transport activity, ongoing efforts are aimed at developing neuron-enriched cultures with minimal glial contamination for these studies. Although one might argue that the regulated trafficking of EAAC1 observed in C6 glioma is an artifact of the cell line, two studies have documented cytoplasmic as well as plasma membrane localization of EAAC1 in brain tissue (11, 12). This cytoplasmic/intracellular localization is unique to the EAAC1 subtype of glutamate transporter (69) and suggests that there is an intracellular pool of EAAC1 that can be redistributed to the cell surface in vivo.

The goals of this study were to determine if receptor-mediated activation of PI3-K increases the activity and cell surface expression of the neuronal glutamate transporter EAAC1 and to determine if this effect is dependent on activation of PKC. Application of PDGF BB increased EAAC1-mediated l-[^3H]glutamate uptake within 30 min and also caused a comparable increase in EAAC1 cell surface expression. The PDGF AA homodimer, a ligand selective for activation of PDGF receptors containing the α subunit, had no significant effect on L-glutamate transport activity. This suggests that the effects observed in the present study are due to activation of receptors containing the β subunit. This result contrasts with an earlier preliminary study of L-aspartate transport in human fibroblasts, which reported an increase in transport activity after PDGF AA but not PDGF BB treatment (61). The transporter subtype regulated by PDGF AA and the mechanism mediating the increase in activity were not identified in this earlier study.

Three lines of evidence reported in the present study suggest that the effects of PDGF were mediated by PI3-K. First, several growth factors mediate a variety of cellular responses in C6 glioma, including NGF, EGF, PDGF, and insulin (47–51). Each of these growth factors have been directly demonstrated to activate the same major signaling pathways, PLC-γ, PI3-K, and MAP kinase, in a number of different cellular systems (52, 57, 58, 62). However, in the present study, only PDGF stimulated PI3-K activity, and only PDGF altered EAAC1-mediated transport activity and cell surface expression. The lack of PI3-K stimulation by the other growth factors tested implies that these receptors couple to different signaling pathways in C6 glioma that do not contribute to the regulation of EAAC1 activity. Second, the effects of PDGF on EAAC1-mediated glutamate uptake activity and cell surface expression were blocked by two different PI3-K inhibitors. Finally, the effects of PDGF were not blocked by inhibitors of other signaling pathways potentially activated by PDGF. These studies suggest that PKC and PI3-K independently regulate cell surface expression of EAAC1 and provide one of the first examples of growth factor-mediated trafficking of neurotransmitter transporters.

The C6 glioma cell line was selected as a model system for these experiments because of its endogenous and selective expression of EAAC1, allowing examination of the transporter in isolation. C6 glioma are an undifferentiated cell line of central nervous system origin that express both neuronal (glutamic acid decarboxylase) and glial (glutamine synthetase and glial fibrillary acidic protein) markers (63–65). Although neurons in

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2. K. Sims and M. Robinson, unpublished observation.
and reduced the effects of PDGF to similar levels below control. In contrast, LY 294002 alone did not affect basal glutamate transport or cause a reduction of cell surface immunoreactivity. Both wortmannin and LY 294002 decreased PI3-K activity to undetectable levels and blocked PDGF stimulation of PI3-K activity. The differential effect of wortmannin on EAAC1 activity and cell surface expression might be attributable to inhibition of alternate kinase targets, but the concentrations used in the present study are below the 200–300 nM IC50 values required for inhibition of these alternate targets (myosin light chain kinase and MAP kinase) (55). Furthermore, we demonstrated in an earlier study that wortmannin inhibits basal EAAC1-mediated uptake with an IC50 value of 15 nM, which is nearly identical to the IC50 value for inhibition of PI3-K (27). Wortmannin and LY 294002 both interact with the p110 subunit of PI3-K but have different mechanisms of action (wortmannin is an irreversible inhibitor and LY 294002 is reversible) (55, 56). Although it is possible that this difference in mechanism may account for the selective effects of wortmannin, it seems more likely that this effect on baseline activity and cell surface expression is related to inhibition of a wortmannin-sensitive, LY 294002-insensitive isoform of PI3-K. Several new isoforms of PI3-K have been identified recently, but their sensitivities to wortmannin and LY 294002 have not been systematically evaluated (for review see Ref. 76). There are some isoforms that display differences in sensitivity to these inhibitors when compared with the "classical" mammalian PI3-K, p85/p110 PI3-K (77–79). Therefore, it is possible that multiple isoforms of PI3-K regulate different aspects of transporter trafficking. For example, a wortmannin-sensitive, LY 294002-insensitive PI3-K may be required for recycling of EAAC1 transporters through an intracellular compartment.

In the present study, we also found that the effects of PDGF were not blocked by the PKC antagonist BII, and the stimulatory effects of PDGF and PMA were not additive. This could imply that both PKC and PI3-K increase EAAC1 activity through independent but converging pathways. Activation of PKC with phorbol esters also increases activity and/or cell surface expression of the GLUT4 glucose transporter (80–82). The somewhat nonspecific PKC inhibitor, staurosporine, blocks the effects of phorbol esters and insulin on glucose uptake with different IC50 values, suggesting phorbol esters and insulin utilize different but possibly converging signaling pathways (81). Because PKC did not activate PI3-K in our system, it seems most likely that these pathways converge downstream of PI3-K. The PKC and PDGF/PI3-K pathways may independently regulate the same limited intracellular pool of transporters. Alternatively, it is possible that a PMA-sensitive, classical PKC activates a downstream effector of PI3-K. Both atypical PKC isoforms and Akt/PKB have been implicated as downstream effectors of PI3-K during insulin-mediated regulation of GLUT4. Expression of constitutively active/dominant negative constructs of both the atypical PKC isoform, PKCζ, and Akt/PKB (83–85) influence insulin regulation of GLUT4 transport and cell surface expression. Molecular biological approaches also suggest that PKCα and PKCε may be downstream effectors of PI3-K signaling (86, 87). At present, it is not known if these kinases can be regulated by PMA-activated PKCs nor is it known if these signaling molecules contribute to the regulation of EAAC1 trafficking.

At present, it is not known if the effects of PDGF and phorbol ester on transport activity and cell surface expression are dependent upon direct phosphorylation of EAAC1 or indirectly mediated through phosphorylation of other proteins required for trafficking of the transporters to and from the cell surface. Recent studies have demonstrated that phorbol ester-induced decreases in GLAST-mediated transport activity are correlated with transporter phosphorylation, but this phosphorylation does not appear to be occurring at a PKC phosphorylation site consensus sequence (88). Earlier studies have demonstrated that GLUT-1 is also phosphorylated by phorbol esters (89). In both of these examples of glutamate transporter phosphorylation, it has not been determined if the changes in transport activity are correlated with changes in transporter cell surface expression. More recently, it has been shown that phosphorylation of the serotonin transporter results in internalization of the transporter and a corresponding reduction in serotonin transport (29). Therefore, although there is currently no evidence that the effects of PDGF or phorbol ester are related to phosphorylation of the EAAC1, it is possible that the redistribution of EAAC1 could involve direct transporter phosphorylation.

Rapid regulation of EAAC1 may be important for regulating renal reabsorption of acidic amino acids and may be critical for proper synaptic transmission and the prevention of excitotoxic injury in the brain. PDGF may represent an endogenous physiologic regulator of neuronal EAAC1 function and glumatergic transmission, because PDGF β receptors and B-chains are expressed in neurons throughout the central nervous system and are enriched in the hippocampus, an area of high EAAC1 expression levels (90, 91). PDGF BB inhibits both GABAβ-dependent inhibitory post-synaptic currents and N-methyl-D-aspartate-dependent excitatory post-synaptic currents (92, 93), suggesting a role in the regulation of rapid synaptic events. Both PDGF B and β receptor mRNA and immunoreactivity are increased after induction of neocortical focal ischemia (94, 95), and PDGF BB pretreatment reduces delayed hippocampal CA1 pyramidal neuron death in a global forebrain ischemia model (96). Although the mechanism of PDGF neuroprotection was not examined in these models, possible mechanisms have been studied in other models. In cortical and hippocampal neuronal cultures exposed to two different models of excitotoxic insult (glucose deprivation or FeSO4), PDGF AA or BB are neuroprotective (97). Importantly, PDGF was effective when applied before, during, or up to 4 h after the onset of the insult, implying that rapid regulatory events contribute to this protection. Prevention of neuronal death was correlated with increases in the activities of catalase, superoxide dismutase, and glutathione peroxidase, suggesting that activation of these enzymes contributes to this effect. The rapid, PDGF-mediated increase in EAAC1 activity and cell surface expression we describe in this study may represent a novel mechanism that contributes to the neuroprotective effects of this growth factor.

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REFERENCES

1. Faden, A. I., Demediuk, P., Panter, S. S., and Vink, R. (1989) Science 244, 798–800
2. Benveniste, H., Drejer, J., Schousboe, A., and Diemer, N. H. (1984) J. Neurochem. 43, 1369–1374
3. Drejer, J., Benveniste, H., Diemer, N. H., and Schousboe, A. (1985) J. Neurochem. 45, 145–151
4. Attwell, D., Barbour, B., and Szakowski, M. (1993) Neuron 11, 461–467
5. Longuemare, M. C., and Swanson, R. A. (1995) J. Neurosci. Res. 40, 379–386
6. Pines, G., Danbolt, N. C., Bjerås, M., Zhang, Y., Bendahan, A., Eide, L., Kneepkl, H., Storm-Mathisen, J., Seeberg, E., and Kanner, B. L. (1992) Nature 360, 464–467
7. Storek, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10955–10959
8. Kainul, Y. and Hediger, M. A. (1992) Nature 360, 467–471
9. Fairman, W. A., Vandenberg, R. J., Arriaza, J. L., Kavanga, M. P., and Amara, S. G. (1995) Nature 375, 599–603
10. Arriaza, J. L., Elasof, S., Kavanga, M. P., and Amara, S. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4155–4160
11. Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., and Kand, R. W. (1994) Neuron 13, 713–725
12. Shashidhara, P., Huntley, G. W., Murray, J. M., Buka, A., Moran, T., Walsh,
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