The neuronal glutamate transporter, EAAC1, appears to both limit spillover between excitatory synapses and provide precursor for the synthesis of the inhibitory neurotransmitter, γ-aminobutyric acid. There is evidence for a large intracellular pool of EAAC1 from which transporter is redistributed to the cell surface following activation of protein kinase C (PKC) or platelet-derived growth factor (PDGF) receptor by seemingly independent pathways. A variety of biotinylation strategies were employed to measure trafficking of EAAC1 to and from the plasma membrane and to examine the effects of phorbol ester and PDGF on these events. Biotinylation of cell surface protein under trafficking-permissive conditions (37 °C) resulted in a 2-fold increase in the amount of biotinylated EAAC1 within 15 min in C6 glioma and in primary neuronal cultures, suggesting that EAAC1 has a half-life of ~5–7 min for residence at the plasma membrane. Both phorbol ester and PDGF increased the amount of transporter labeled under these conditions. Using a reversible biotinylation strategy, a similarly rapid internalization of EAAC1 was observed in C6 glioma. Phorbol ester, but not PDGF, blocked this measure of internalization. Incubation at 18 °C, which blocks some forms of intracellular membrane trafficking, inhibited PKC- and PDGF-dependent redistribution of EAAC1 but had no effect on basal trafficking of EAAC1. These studies suggest that both PKC and PDGF accelerate delivery of EAAC1 to the cell surface and that PKC has an additional effect on endocytosis. The data also suggest that basal and regulated pools of EAAC1 exist in distinct compartments.

A family of Na\(^{+}\)-dependent transporters both ensures appropriate excitatory signaling and limits the excitotoxic potential of glutamate in the mammalian central nervous system. This family includes members that are generally expressed in astrocytes (GLT-1 and GLAST) or neurons (EAAC1 and EAAT4) (for reviews, see Refs. 1–3). EAAC1 is enriched on the postsynaptic processes of pyramidal cells in cortex and hippocampus, two pathways that display remarkable synaptic plasticity and exquisite sensitivity to excitotoxic insults (4, 5). There is evidence that EAAC1 limits spillover between excitatory synapses in hippocampus (6, 7). EAAC1 is also found on inhibitory interneurons (8) where it appears to provide precursor for the synthesis of the inhibitory neurotransmitter, γ-aminobutyric acid (9). Antisense oligonucleotide knockdown of EAAC1 disrupts γ-aminobutyric acid synthesis and causes an epilepsy-like phenotype (10). In addition to these physiologic roles, there is evidence that EAAC1 expression is altered under various pathologic conditions (11–14).

The activities of the GLUT4 subtype of glucose transporter and of several neurotransmitter transporters can be rapidly altered by changing the number of transporters present at the plasma membrane (for reviews, see Refs. 15–18). In both C6 glioma and primary neuronal cultures, less than 30% of total EAAC1 is found on the cell surface, with the remainder on intracellular vesicles of unknown identity (19, 20). Similarly, there is evidence for an intracellular pool of EAAC1 in vivo (8). In C6 glioma, activation of PKC\(^{1}\) with PMA causes a rapid (within minutes) increase in EAAC1-mediated activity (21). This effect is associated with a ~2-fold increase in cell surface EAAC1 that is independent of synthesis of new transporters but is instead related to a redistribution of EAAC1 from a subcellular compartment to the cell surface (22). PDGF also causes an increase in both EAAC1-mediated activity and cell surface expression in C6 glioma that is blocked with inhibitors of PI3K. These effects of PDGF are not blocked by PKC antagonists, suggesting that two independent signaling pathways regulate EAAC1 trafficking (19). Transfection of C6 glioma with the neurotensin receptor subtype 1 and activation of this receptor also cause a redistribution of EAAC1 that is not blocked by inhibitors of either PKC or PI3K (23). There is evidence for an association between regulated trafficking of EAAC1 and models of learning and memory. Levenson and coworkers (24) have shown a redistribution of EAAC1 from an intracellular pool to the cell surface in both long term potentiation and contextual fear conditioning. However, the mechanisms by which these events regulate EAAC1 trafficking have not been identified.

To begin to define the mechanisms by which PMA and PDGF might regulate EAAC1 cell surface expression, we examined...
the kinetics of EAAC1 trafficking to or from the cell surface and the effects of phorbol ester or PDGF on these events. Here we report that there is robust basal trafficking of the transporter and that both PMA and PDGF increased the delivery of EAAC1 to the membrane. PMA, but not PDGF, also inhibited net internalization of EAAC1. At a lower temperature (18 °C), basal delivery of EAAC1 to the plasma membrane was unaffected, but a temperature increase did not, and this effect was blocked. Basal delivery of EAAC1 to the plasma membrane was comparably rapid in primary neuronal cultures, where the cellular milieu is more likely to mimic that observed in vivo. Based on these findings, we conclude that: (i) EAAC1 trafficking to and from the cell surface in both C6 and neurons is rapid compared with many other membrane proteins; (ii) although both PKC and PDGF increase cell surface expression, they appear to have differential effects on delivery and internalization of the transporter; and (iii) basal and regulated delivery of EAAC1 to the plasma membrane may originate from distinct intracellular pools.

**EXPERIMENTAL PROCEDURES**

**Materials**—C6 glioma were obtained from the American Type Culture Collection (Rockville, MD). DMEM, trypsin-EDTA, Neurobasal medium, B27 supplement, t-glutamine, and penicillin-streptomycin were purchased from Invitrogen. Fetal bovine serum was purchased from Amersham Biosciences. Monoclonal anti-transferrin receptor antibody was purchased from Zymed Laboratories Inc. BSA, MesNa, PMA, and the bicinchoninic acid (BCA) protein assay kit were purchased from Pierce. UltraLink immobilized monomeric avidin, NHS-SS-biotin, UltraLink monomeric avidin-coated Sepharose beads solution. In addition, an aliquot was mixed with an equal volume of SDS-PAGE (4%) loading buffer and labeled as the “lyase” fraction. Another aliquot (300 μl) was incubated overnight with 250 μl of UltraLink monomeric avidin-coated Sepharose beads solution. After centrifugation for 15 min, an aliquot of the resulting supernatant was diluted into an equal volume of (1%) loading buffer and labeled as the “non-biotinylated” fraction. The beads were washed twice with radioimmunoprecipitation assay buffer, and then sequentially with a “high-salt” (50 mM Tris, 5 mM EDTA, 500 mM NaCl, 0.1% Triton X-100, pH 7.5) solution, and a “low-salt” solution (50 mM Tris, pH 7.5), with centrifugation between each wash as per the manufacturer’s instructions. The beads were then incubated for 10 min at room temperature in 100 μl of PBS (pH 7.4) followed by washing with PBS (pH 7.4). Then, the beads were incubated with biotinylated proteins for 30 min at 37 °C. After centrifugation, the resulting supernatant was collected as the “biotinylated” fraction.

**Delivery of EAAC1 to the Plasma Membrane**—Biotinylation under trafficking-permissive conditions was carried out as follows. C6 glioma or primary neurons were rinsed twice with PBS/Ca2+/Mg2+ solution at 37 °C and then cell surface proteins were labeled with the reversible biotinylating reagent NHS-S-S-biotin (2 ml of 1 mg/ml). After 30 min, excess biotinylating reagent was quenched by rinsing the cells twice with PBS/Ca2+/Mg2+ solution containing 100 mM glycine, and incubation in PBS/Ca2+/Mg2+/glycine for 30 min at 4 °C with gentle shaking. Cells were then rinsed twice with PBS/Ca2+/Mg2+ before being lysed in 1 ml of radioimmunoprecipitation assay buffer containing protease inhibitors for 30 min. The cells were scraped from the plates, and the lysates were centrifuged for 15 min at 12,500 rpm. After removal of the cellular debris, an aliquot of lysate was frozen for western blot analysis. The cells were treated with either PMA or PDGF during biotinylation and that both PMA and PDGF increased the delivery of EAAC1 to the plasma membrane was unaffected, and then incubated with 4 ml of biotinylation solution at the same temperature for different periods of time. In some experiments, cells were treated with either PMA or PDGF during biotinylation. After rinsing cells into ice-cold PBS/Ca2+/Mg2+ solution containing glycine to stop trafficking, biotinylated proteins were extracted as described above.

**Internalization of EAAC1**—Reversible biotinylation was performed as previously described (26). After the preincubation with DMEM/BSA solution, cells were rinsed twice with PBS/Ca2+/Mg2+ solution at 4 °C and then cell surface proteins were labeled with the reversible biotinylating reagent NHS-S-S-biotin (2 ml of 1 mg/ml). After 30 min, excess biotinylating reagent was quenched with PBS/Ca2+/Mg2+ solution containing glycine as described above. Cells were rapidly rinsed twice with pre-warmed (37 °C) plain DMEM and incubated for varying periods of time. To halt internalization, cells were rinsed twice with ice-cold sodium-tris (NT) buffer (150 mM NaCl, 1 mM EDTA, 0.2% BSA, 20 mM Tris, pH 8.6) followed by an incubation for an additional 10 min. Cell surface-bound biotinylating reagent was stripped by incubating the cells twice for 5 min in PBS/Ca2+/Mg2+ buffer containing 1 mg/ml of MesNa. MesNa was only used for 6 months after date of purchase. In each experiment, one plate labeled “No MesNa” did not undergo re-warming or subsequent stripping of biotinylating reagent to provide a measure of the total pool of cell surface transporter available for internalization. A second plate, labeled “t = 0,” did not undergo re-warming before having been washed with ice-cold buffer containing the reagent, allowing us to measure the efficiency of the MesNa reagent. Both of these controls were rinsed into NT buffer, and all stripping occurred at the same time. After removal of cell surface-bound biotinylating reagent, cells were rinsed twice with PBS/Ca2+/Mg2+ buffer, and biotinylated proteins were extracted as described above.

**Western Blot Analysis**—Aliquots of lysate from individual samples containing equal amounts of protein (10–20 μg) were loaded in an 8% SDS-polyacrylamide gel. Equal volumes of all three fractions were analyzed, such that if the yield from the extraction of biotinylated proteins was 100%, the sum of the amount of immunoreactivity in the
non-biotinylated and biotinylated fractions should equal the amount of immunoreactivity in the lysate. When the number of samples exceeded the number of lanes available in a single mini-gel, then two gels were prepared with aliquots of lysate from each sample present in both gels. Proteins were transferred to polyvinylidene difluoride membranes. These membranes were blocked with Tris buffer containing (0.1%) Tween 20 and 1% nonfat milk and probed with specific antibodies to EAAC1 (1:75), GLT-1 (1:10,000), or transferrin receptor (TfR: 1 µg/ml). All blots were also probed with an anti-actin antibody (1:5,000) to control for possible cell lysis prior to biotinylation. Proteins were visualized using ECL as per the manufacturer’s instructions.

Immunoreactivity was quantified using National Institutes of Health Image software (available at rsb.info.nih.gov/nih-image/). In some cases, different exposures of the film were used to quantify different immunoreactive bands to ensure that the signal was within the linear range. Immunoreactivity of each sample was normalized to the amount of actin in the lysate fraction of that sample from the same gel and expressed as a percentage of the amount of immunoreactivity observed in the control sample within each fraction. Data are presented as the (mean ± S.E. of the mean) and compared using ANOVA.

RESULTS

Time Course for PMA- and PDGF-induced Increases in Cell Surface EAAC1—Before determining if PMA or PDGF affect the rates of delivery or removal of EAAC1 from cell surface, it was necessary to examine the time course for the effects of these agents on cell surface expression. C6 glioma, a brain-derived cell line that endogenously expresses EAAC1 and none of the other Na+−dependent glutamate transporters (22), were treated with the phorbol ester, PMA, for different periods of time. Cell surface expression of EAAC1 was examined using a membrane-impermeant biotinylating reagent followed by batch extraction of the biotinylated proteins with avidin-coated beads. Under control conditions, −20% of EAAC1 was found in the biotinylated fraction; this is consistent with the large pool of intracellular immunoreactivity observed with confocal microscopy (20, 22). Under identical conditions, the same biotinylating reagent captures greater than 80% of the homologous transporter, GLT-1, providing evidence that the reaction conditions are appropriate for efficient labeling of cell surface proteins (27). PMA caused a significant increase in EAAC1 cell surface expression within 2 min and a nearly 2-fold increase in cell surface expression within 15 min (Fig. 1, A and B). The change in surface expression was maximal at 15 min and was not significantly different from control with 60 min of treatment. PDGF increased cell surface expression of EAAC1 with a similar time-course having a maximal effect at 15 min.2 Notably, neither PMA nor PDGF caused a complete redistribution of EAAC1 from the intracellular pools to the cell surface. It is not known if this observation is related to “desensitization/down-regulation” of the signaling pathway or depletion of a specific “regulated pool” of EAAC1 that can be redistributed to the cell surface. These studies identify 15 min as a critical time for determining if PMA or PDGF affect delivery or removal of EAAC1 from the cell surface.

Rate of Delivery of EAAC1 to the Cell Surface under Basal Conditions—Other membrane proteins, including the TfR, DAT, GAT-1, the choline transporter, and GLUT4, have been reported to recycle rapidly to and from the cell surface (for reviews, see Refs. 15, 18, 28). To measure the rate of delivery of EAAC1 to the cell surface, C6 glioma were incubated under trafficking permissive conditions (37 °C) in the presence of the biotinylating reagent. Under these conditions, the membrane-impermeant biotinylating reagent should label transporters that cycle through the plasma membrane. In each experiment, the amount of EAAC1 that was biotinylated under conditions not permissive to trafficking (4 °C) was also examined, and the amount of transporter delivered to the cell surface was expressed as a percentage of this steady-state measure of transporter surface expression. At 37 °C, there was a time-dependent increase in the amount of biotinylated protein (Fig. 2, A and B). There was no evidence for changes in the amount of biotinylated actin, providing evidence that this increase in biotinylated EAAC1 cannot be attributed to labeling of intracellular pools of EAAC1. The increase in biotinylated EAAC1 was accompanied by a decrease in the amount of transporter in the non-biotinylated fraction and no change in the total amount of EAAC1 immunoreactivity in the cell lysates (Fig. 2, A and B). Within 15 min, there was nearly a 2-fold increase in the amount of biotinylated EAAC1, implying that the entire population of cell surface transporter was replaced over this time period to maintain the steady-state level of cell surface EAAC1. There was a continued increase in the amount of biotinylated transporter at 30 and 60 min consistent with this rapid rate of turnover of cell surface EAAC1. To test the validity of this approach, the rate of delivery of TfR was examined. Consistent with previous studies in other cellular systems, the amount of biotinylated TfR increased in a time-dependent manner with nearly the entire population being labeled within 30 min (Fig. 2C). Although the acylation reaction between the activated carboxylic acid (NHS-ester) of the biotinylating reagent and the primary amine (typically lysine) from a cell surface protein is thought to be quite fast (29, 30), labeling of transporter that

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2 A. L. Sheldon and M. B. Robinson, unpublished observations.
cycles through the plasma membrane may not be 100% efficient. Therefore, these values may represent an underestimate of the rate of delivery/removal of transporter to and from the cell surface under basal conditions but are consistent with a half-life for turnover of no longer than ∼5–7 min.

**Rate of Internalization of EAAC1—**As a complementary approach, EAAC1 internalization was examined using a reversible biotinylation strategy. C6 glioma cells were incubated with a disulfide bond-containing biotinylation reagent at 4 °C to label cell surface proteins. After re-warming to trafficking-permissive conditions for various periods of time, cells were cooled and cell surface-bound biotinylated transporter was removed by treatment with MesNa, a membrane-impermeant reducing agent. Using this approach, 21.5 ± 6.9% of EAAC1 was biotinylated at steady state, consistent with our other studies; this sets the upper limit for the amount of transporter that could become inaccessible to MesNa (31). With no re-warming, slightly more than 80% of the biotinylating reagent can be stripped (Fig. 3, A and B; 0 min at 37 °C before MesNa). The 20% of biotinylating reagent that cannot be removed with MesNa is presumably related to incomplete stripping of cell surface biotinylating reagent by MesNa, but may also be related to inaccessibility of this pool to MesNa (31). With this approach, we observed a time-dependent increase in the amount of biotinylated transporter that became inaccessible to MesNa (Fig. 3, A and B). In contrast to the half-life estimated from measurement of delivery of transporter to the cell surface, only slightly greater than 50% of the biotinylated EAAC1 appeared to be internalized within 15 min. It is possible that internalized transporter rapidly recycles, that C6 glioma contain a reducing environment that cleaves the disulfide bond in the spacer arm of the biotinylating reagent, or that having only 20% of the transporter on the cell surface under steady state limits detection of internalized transporter at early time points.

To address these possibilities, C6 glioma cells were treated with PMA to increase the pool of EAAC1 available for internalization from the cell surface. After incubation with PMA for 15 min, cells underwent biotinylation and quenching of excess biotinylating reagent at 4 °C, providing a substantial opportunity for washout of PMA without allowing the transporter to re-internalize. Under these conditions, there was a robust increase in the amount of transporter that was inaccessible to MesNa within 2 min, and nearly 50% of EAAC1 was inaccessible within 5 min (Fig. 4). There was no further increase in the amount of transporter that was inaccessible to MesNa with longer incubations, suggesting that internalized transporter is rapidly recycled. Although these studies assume that PMA...
does not have residual effects on the rates of membrane trafficking, this rate of internalization is consistent with a half-life of ~5-7 min.

PMA and PDGF Increase Delivery of EAAC1 to the Cell Surface—Having observed a rapid trafficking of EAAC1, we next sought to determine if PMA and PDGF influence the rate at which EAAC1 is delivered to the plasma membrane. C6 glioma cells were treated with vehicle, PMA (100 nm), or PDGF (10 μg/ml) at 37 °C in the presence of biotinylating reagent for 15 min (Fig. 5). As was observed in the studies presented in Fig. 2, there was an increase of ~2-fold in the amount of biotinylated EAAC1, implying that the number of transporters delivered to the plasma membrane during this time was equal to the number of transporters that exist on the cell surface at steady state. Both PMA and PDGF significantly increased the amount of transporter biotinylated during this time, providing strong evidence that they caused the delivery of additional EAAC1 to the plasma membrane. In parallel studies, PMA increased steady-state levels of EAAC1 to 194 ± 11% of control, and PDGF increased steady-state cell surface EAAC1 to 143 ± 8% of control (measured with biotinylation after treatment). It should be noted that the relative magnitude of these effects was reversed in the two assays: PDGF had a larger effect on delivery, whereas PMA had a larger effect on steady-state EAAC1. This suggests that the effects of PMA cannot simply be attributed to accelerated delivery of EAAC1.

PMA, but Not PDGF, Inhibits the Appearance of an Internal Pool of Biotinylated EAAC1—To determine if PMA and/or PDGF inhibit the internalization of EAAC1 from the cell membrane, C6 glioma were incubated with a biotinylating reagent for 4 h at 37 °C. Cells were re-warmed to 37 °C and treated with vehicle, PMA (100 nm), or PDGF (10 μg/ml) for 15 min (Fig. 6). In parallel studies, PMA increased steady-state levels of EAAC1 to 205 ± 29% of control, and PDGF increased steady-state cell surface EAAC1 to 141 ± 13% of control (measured with biotinylation after treatment). Following re-warming and stripping of cell surface biotinylating reagent with MesNa, the amount of biotinylated EAAC1 in vehicle-treated cells was comparable to that observed in Fig. 3. Although PDGF had no effect on the amount of transporter that became inaccessible to MesNa, PMA completely blocked the appearance of an internal pool of biotinylated EAAC1. This provides strong evidence for a differential effect of PDGF and PMA on EAAC1 trafficking and suggests that PMA reduces internalization of EAAC1.

Lowered Assay Temperature Inhibits Both PMA- and PDGF-induced Redistribution of EAAC1—In several cellular systems, lowered temperatures (15-20 °C) attenuate exit of proteins from the late endosome back to the plasma membrane, without affecting endocytosis of cell surface proteins (26, 32, 33). This phenomenon has been well studied for the constitutive recycling of the TfR. If the pool of EAAC1 that is mobilized by PMA and PDGF resides in a trans-endosomal pathway, then we might expect that incubation at 18 °C would attenuate or block the effects of PMA or PDGF, respectively. C6 glioma were treated with vehicle, PMA, or PDGF for 15 min at either 37 °C or 18 °C before undergoing biotinylation at 4 °C. Consistent with our earlier studies, both PMA and PDGF significantly increased the amount of EAAC1 on the cell surface when cells were treated at 37 °C (Fig. 7, A and B). However, at 18 °C, neither PMA nor PDGF significantly increased the amount of EAAC1 at the plasma membrane compared with vehicle-treated cells. As a complementary approach, C6 glioma cells were treated at either 37 °C or 18 °C with PDGF, and Na+-dependent glutamate uptake activity was measured at 37 °C. At 37 °C, PDGF increased EAAC1-mediated activity (Fig. 7C), but
PDGF had no effect on activity when cells were treated at 18 °C, consistent with the biotinylation experiments. Although we considered performing similar experiments with PMA, there are data to suggest that PKC has effects on both cell surface expression and intrinsic activity of EAAC1 (20), which would confound these studies.

The steady-state levels of many membrane proteins at the plasma membrane are decreased by incubation at 18 °C because of the preferential inhibition of delivery relative to internalization. In our studies, incubation at 18 °C did not decrease either EAAC1 cell surface expression or transporter activity (Fig. 7). Therefore, it seemed possible that constitutive trafficking of EAAC1 might be insensitive to incubation at 18 °C. To address this possibility, the rate of delivery of EAAC1 to the plasma membrane was examined by incubating C6 glioma in the presence of biotinylating reagent at 18 °C. As was observed at 37 °C in Fig. 2, there was a marked time-dependent increase in biotinylated EAAC1, implying that constitutive trafficking is not inhibited at 18 °C (Fig. 8A). Because TfR delivery is affected by 18 °C in other cell systems, the effects of incubation at 18 °C on steady-state levels of TfR were examined to confirm that classic endosomal pathways are temperature-sensitive in C6 glioma. As would be expected for the selective inhibition of exit of proteins from the late endosome back to the plasma membrane, the level of TfR decreased to 64 ± 7% of control (mean ± S.E., n = 4) after 15 min at 18 °C (Fig. 8B). These studies provide strong evidence that delivery of TfR to the cell surface is attenuated at this temperature in C6 glioma. To determine if incubation at 18 °C was simply inhibiting PMA-induced PKC activation, C6 glioma were transfected with GLT-1. In this system, PKC activation causes a loss of GLT-1 from the cell surface and with longer incubations a loss of transporter immunoreactivity (27, 34). As was previously observed, PMA caused a loss of GLT-1 immunoreactivity in the biotinylated fraction (58% mean of two experiments within 20%) at 37 °C and a comparable decrease (63% mean of two experiments within 10%) at 18 °C (Fig. 8C). These results suggest that the lack of effect PMA on cell surface expression of EAAC1 at 18 °C cannot simply be attributed to a generalized inhibition of PKC-dependent signaling. Together, these studies show that incubation at 18 °C selectivity inhibits PMA- or PDGF-induced redistribution of EAAC1 from a subcellular compartment to the cell surface but does not inhibit constitutive recycling of EAAC1, implying that there may be distinct pools of EAAC1 (recycling and regulated).

Delivery of EAAC1 to the Cell Membrane in Primary Neuronal Cultures—These studies strongly suggest that EAAC1 is rapidly trafficked to and from the cell surface and that these steps are differentially regulated in C6 glioma. However, it is not clear if similar events occur in a system that has a cellular milieu that is presumably more like that observed in vivo. To measure basal insertion of EAAC1 in neuronal cultures, primary cortical neurons were incubated in the presence of bio-

![Fig. 6. Effects of PMA and PDGF on EAAC1 internalization. C6 glioma cells were biotinylated with a disulfide-containing biotinylating reagent under conditions that prevent trafficking (4 °C). A subset of plates were then re-warmed to 37 °C for 15 min (≥) 100 nM PMA or 10 μg/ml PDGF before undergoing stripping with MesNa as described under “Experimental Procedures.” A, representative Western blot of the biotinylated fraction. See the legend to Fig. 3 for an explanation of the labels. B, summary of data from four to six independent experiments (mean ± S.E.). **, p < 0.01 compared with 0 min; ##, p < 0.01 compared with 15 min; by one-way ANOVA with Bonferroni post-hoc analysis.](http://www.jbc.org/)

![Fig. 7. Effects of PMA and PDGF on EAAC1 cell surface expression and uptake activity at 37 °C and 18 °C. C6 glioma were treated for 15 min ± 100 nM PMA or 10 μg/ml PDGF at either 37 °C or 18 °C. Cells were then biotinylated at 4 °C and processed as described under “Experimental Procedures.” A, representative Western blot showing the effects of PMA and PDGF on biotinylated EAAC1. B, data from three (PDGF) or six (PMA) independent experiments are summarized (mean ± S.E.) and expressed as a percentage of vehicle-treated (VEH) at 37 °C. ***, p < 0.001; *, p < 0.05 compared with 37 °C/VEH by one-way ANOVA with Bonferroni post-hoc analysis. C, Na+-dependent glutamate transport activity in C6 glioma measured at 18 °C after treatment as described in A and B. Data are the mean ± S.E. of three independent experiments.](http://www.jbc.org/)
Expression. This experiment has been repeated once.

Western blot of the effects of PMA (18°C) for increasing periods of time and compared with the steady-state level of EAAC1 measured at 4°C. We observed a time-dependent increase in biotinylated EAAC1 and complementary decrease in non-biotinylated EAAC1 with no change in biotinylated actin.

FIG. 8. Effects of 18°C on constitutive delivery of EAAC1, recycling of TFR, and PMA-induced redistribution of GLT-1. A, representative immunoblot of the increase in biotinylated EAAC1 observed when C6 glioma were incubated at 18°C in the presence of biotinylating reagent for different periods of time. There were no increases in the amount of biotinylated actin. This experiment has been repeated two additional times with similar results. The mean increase in biotinylated EAAC1 at 15 min from three independent experiments is 211 ± 14% (mean ± S.E.). B, analysis of steady-state TFR immunoreactivity in experiments presented in Fig. 7, A and B. C, representative Western blot of the effects of PMA (15°C or 37°C) on GLT1 cell surface expression. This experiment has been repeated once.

tinylation reagent at 37°C. Under these conditions, there was a rapid time-dependent increase in the amount of biotinylated transporter with a 209 ± 15% (n = 6) increase at 15 min, which is comparable to that observed in C6 glioma (Fig. 9). As has been previously observed (20), PMA increased cell surface expression of EAAC1 in the cortical neurons (146 ± 7% of control, n = 6). We also observed that PDGF caused an increase in the steady-state level of cell surface EAAC1 (150 ± 16% of control). PMA had a modest effect on delivery of EAAC1 to the cell surface (vehicle 203 ± 16% of control compared with 237 ± 23% in PMA-treated cultures, n = 6). Although this effect was not statistically significant by ANOVA, in six of six experiments the amount of transporter biotinylated under trafficking permissive conditions was greater in the presence of PMA than in the absence of PMA. In two of three preliminary experiments, PDGF modestly increased the amount of transporter biotinylated under trafficking permissive conditions (data not shown).

DISCUSSION

Regulation of cell surface expression of neurotransmitter transporters is a shared mechanism associated with rapid (within minutes) changes in function that can occur without the delay associated with protein synthesis. The pre-synaptic choline and norepinephrine transporters are rapidly redistributed to the plasma membrane in response to depolarization (35, 36). Activation of PKC decreases cell surface levels of several transporters, including DAT, GAT-1, NET, SERT, and the GLT-1 subtype of glutamate transporter (for reviews see Refs. 16, 18, 37, and 38). It appears that activation of PKC accelerates the rate of removal of the transporters from the plasma membrane (NET, GAT-1, and DAT), but it also appears that activation of PKC slows the rate of delivery of DAT to the plasma membrane (26, 39, 40). This implies that PKC may modify two or more intermediaries to regulate DAT surface expression.

In contrast to the effects on the other neurotransmitter transporters, EAAC1 cell surface expression is increased following activation of PKC (20, 22). Similarly, activation of the PDGF receptor also increases EAAC1 cell surface expression (19). Although the effects of PDGF are blocked by inhibitors of PI3K, they are not blocked by inhibitors of PKC, suggesting that the effects of PDGF are independent of PKC activation. However PMA and PDGF do not have additive effects (19) implying that PMA and PDGF converge on either a common signaling pathway or a finite "regulated" pool of transporter.

The goal of the present study was to determine if PKC or PDGF affect removal or delivery of EAAC1 at the plasma membrane.

Under basal conditions, the rate of delivery of EAAC1 to the plasma membrane was consistent with a half-life for the residence of EAAC1 at the cell surface of 5–7 min. Because capture with biotinylating reagent at the cell surface is not instantaneous, turnover at the plasma membrane may be even faster. A similar rate of appearance of EAAC1 was observed in both C6 glioma and primary neuronal cultures, suggesting that this rapid turnover is not simply related to the cellular milieu in C6 glioma. Although measurements of internalization under basal conditions suggested a slower half-life, this may be due to difficulties associated with detecting internalization of the small amounts (∼20%) of transporter found on the cell surface. In fact, when the cell surface pool of EAAC1 was increased with PMA, we were able to detect rapid internalization consistent with a half-life of ∼5 min. Compared with other neurotransmitter transporters, this rate of turnover of cell surface EAAC1 is as fast as that observed for GAT-1 (39) and at least twice as fast as that observed for DAT or NET (26, 40). With longer incubations, the amount of internalized biotinylated transporter did not continue to increase, suggesting that transporters are rapidly recycled. An earlier study has shown that the half-life of pulse-chase-labeled EAAC1 is ∼6 h in C6 glioma (41); therefore, this inability to capture 100% of the cell surface EAAC1 following internalization cannot be attributed to synthesis of new transporter.

Two separate sets of observations suggest that PMA and PDGF have differential effects on trafficking of EAAC1. Both PMA and PDGF increased the amount of transporter captured with biotinylating reagent at 37°C, demonstrating that they accelerate delivery of EAAC1 to the plasma membrane, but the
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effect of PDGF was greater than the effect of PMA. In contrast, PMA consistently had a larger effect than PDGF on steady-state EAAC1 in our studies (206 ± 15% compared with 141 ± 7%, n = 15). Therefore, it is unlikely that the effects of PMA can simply be explained by accelerated delivery of EAAC1. In fact, PMA, but not PDGF, blocked the appearance of MesNa-inacceivable transporter. The simplest explanation for this observation is that activation of PKC inhibits internalization of the transporter. It is also possible that this effect of PMA could be attributed to accelerated recycling of biotinylated transporters back to the cell surface. Together, these studies suggest that PKC regulates both delivery and removal of EAAC1, whereas PDGF only affects delivery.

Although both PMA and PDGF accelerated delivery of EAAC1 to the plasma membrane, it is not clear if these two signaling pathways converge on a single target. It is possible that these pathways result in direct modification of EAAC1. Alternatively, these signaling pathways may modify proteins on either the EAAC1-containing vesicle or the target region of the plasma membrane to facilitate membrane fusion. Finally, it is possible that these signaling pathways modify cytoskeletal elements required for forward trafficking of EAAC1. Very little is known about the molecules required for recycling of the different neurotransmitter transporters. There is evidence that synaptaxin 1A is required for delivery of the GAT-1, NET, SERT, and the glycine transporter types 1 and 2 to the plasma membrane (42–45). Internalization of DAT, GAT-1, and pre-synaptic choline transporter from the plasma membrane may be dependent on clathrin (39, 46, 47), whereas NET turnover may be dependent on lipid rafts (40). Although EAAC1 is localized to lipid rafts in HEK293 cells, it has not been determined if this pathway is required for endocytosis (48). Although these studies suggest many possible targets for altering transporter trafficking, none have been shown to be the direct targets of post-translational modification by signaling molecules.

To begin to determine the source of EAAC1 that can be distributed to the plasma membrane, assay temperatures were lowered to 18 °C. Multiple studies suggest that incubation at 18 °C inhibits the exit of proteins from the late endosome to the plasma membrane (49–51), and in fact this treatment was recently shown to prevent recycling of DAT (28). Although incubation at 18 °C completely abolished the regulated redistribution of EAAC1, surprisingly it had no effect on basol delivery of transporter to the plasma membrane. These data imply that regulated delivery of EAAC1 is derived from a distinct population of transporters. This idea is supported by the fact that neither PMA nor PDGF cause more than a doubling of cell surface transporter leaving ~60% of EAAC1 within the cell. A similar model has been proposed for the GLUT4 subtype of glucose transporter, which is redistributed to the plasma membrane in response to insulin in adipocytes and skeletal muscle (for reviews, see Refs. 15 and 52). This transporter is thought to be sorted into two distinct compartments with the first compartment feeding constitutive recycling and the second existing as a static secretory pool that can be rapidly mobilized in response to insulin (53–56). Interestingly, as we propose for the effects of PMA on EAAC1 trafficking, it appears that insulin both accelerates delivery of GLUT4 from the secretory pool and inhibits GLUT4 endocytosis (57, 58).

The precise nature of the role of EAAC1 in shaping excitatory synaptic transmission remains largely unknown. However, it is interesting that the regulated and constitutive recycling of EAAC1 displays similarities to that observed for AMPA receptors. As we observed with EAAC1, the turnover of un-anchored AMPA receptors is relatively fast, with ~10–20% turnover occurring in 10 min (59). The number of cell surface AMPA recep-

tors is increased in long term potentiation and decreased in long term depression (for reviews, see Refs. 60–64) and redistribution of EAAC1 to the plasma membrane has been reported in both long term potentiation and contextual fear conditioning (24). Finally, parallel mechanisms may coordinate increase EAAC1 surface expression and decrease GluR2-containing receptors in post-synaptic spines. For example, PKCε is thought to mediate internalization of GluR2 and forms complexes with GluR2 (65); PKCε may also be responsible for redistribution of EAAC1 (20) and forms complexes with EAAC1 in C6 gloma or brain tissue (66). Because EAAC1 is enriched in peri-synaptic regions surrounding the post-synaptic density that contain AMPA receptors (67, 68), the notion that both the transporters and the receptors rapidly recycle and are regulated by similar mechanisms suggests that regulated trafficking of EAAC1 may have a role in synaptic plasticity.

In summary, we provide evidence that EAAC1 is trafficked between an intracellular compartment and the cell surface with a half-life of ~5 min in both C6 gloma and in neurons maintained in culture. Both PMA and PDGF mobilize EAAC1 from an intracellular pool that appears to be distinct from this constitutively trafficking pool. Finally, PMA also appears to inhibit internalization of EAAC1, providing evidence for differential regulation of EAAC1 trafficking by PKC and PI3K.

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