Expression of the GLT-1 Subtype of Na⁺-Dependent Glutamate Transporter: Pharmacological Characterization and Lack of Regulation by Protein Kinase C

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Accepted for publication February 16, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Several subtypes of Na⁺-dependent glutamate transporters have been pharmacologically differentiated in brain tissues. Five distinct cDNA clones that express Na⁺-dependent glutamate transport activity have been isolated. One goal of the current study was to compare the pharmacological properties of the rat GLT-1 subtype of transporter to those identified previously using rat brain tissues. To accomplish this goal, GLT-1 was stably transfected into two different cell lines that express low levels of endogenous transport activity (MCB and L-M (TK-)). Several clones stably transfected with GLT-1 were isolated. In each cell line, Na⁺-dependent glutamate transport activity was saturable with similar \( K_m \) values (19 and 37 \( \mu M \)). The pharmacological properties of GLT-1-mediated transport in these cell lines paralleled those observed for the predominant pharmacology observed in cortical crude synaptosomes.

These data are consistent with other lines of evidence that suggest that GLT-1 may be sufficient to explain most of the Na⁺-dependent glutamate transport activity in cortical synaptosomes. Although recent studies using HeLa cells have suggested that GLT-1 can be rapidly up-regulated by activation of protein kinase C (PKC), modulation of PKC or phosphatase activity had no effect on GLT-1-mediated activity in these transfected cell lines. To determine if GLT-1 regulation by PKC is cell-specific, HeLa cells, which endogenously express the EAAC1 subtype of transporter, were stably transfected with GLT-1. Although EAAC1-mediated activity was increased by activation of PKC, we found no evidence for regulation of GLT-1. Despite the present findings, GLT-1 activity may be regulated by PKC under certain conditions.

The acidic amino acids, glutamate and aspartate, are the predominant excitatory neurotransmitters in the mammalian central nervous system (for review, see Mayer and Westbrook, 1987). In addition to their essential role as mediators of rapid signaling, these excitatory amino acids (EAAs) also contribute to brain damage observed in acute insults to the nervous system, including hypoxemia, head trauma, and seizure disorders (for review, see Choi, 1992). The levels of EAAs approach 10 mmol/kg in the brain whereas extracellular levels are maintained at micromolar or submicromolar concentrations (for review, see Schousboe, 1981). Because there is no evidence for extracellular metabolism, it is generally assumed that low extracellular concentrations of the EAAs are maintained by Na⁺-dependent transport activity (Schousboe, 1981). Malfunction and/or reverse operation of transporter proteins results in an accumulation of extracellular EAAs and excessive activation of EAA receptors contributing to excitotoxicity (for review, see Attwell et al., 1993).

In the early 1990s, three cDNA clones that express Na⁺-dependent glutamate transport in heterologous expression systems were identified and named GLAST (Storck et al., 1992), GLT-1 (Pines et al., 1992), and EAAC1 (Kanai and Hediger, 1992). Using strategies based on the homology of these transporters, three human homologs were identified and named EAAT1–3 (Arriza et al., 1994) and then subsequently two additional glutamate transporters (neuronal EAAT4 and retinal EAAT5) were identified (Fairman et al., 1995; Arriza et al., 1997).

Before the cloning of individual transporters, pharmacological studies provided evidence for multiple subtypes of transporters that are differentially expressed in various brain regions (for review, see Robinson and Dowd, 1997). For example, transport in crude synaptosomal membranes prepared from forebrain (cortex, hippocampus, or striatum) re-

ABBREVIATIONS: Bis, bisindolylmaleimide II; DHK, dihydrokainate; EAA, excitatory amino acid; L-AAD, L-\( \alpha \)-aminoadipate; L-AP3, L-2-amino-3-phosphonopropionate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; L-trans-PDC, L-trans-pyrrolidine-2,4-dicarboxylate; mGluR, metabotropic glutamate receptor; DMSO, dimethyl sulfoxide.

Received for publication August 26, 1998.

These data are consistent with other lines of evidence that suggest that GLT-1 may be sufficient to explain most of the Na⁺-dependent glutamate transport activity in cortical synaptosomes. Although recent studies using HeLa cells have suggested that GLT-1 can be rapidly up-regulated by activation of protein kinase C (PKC), modulation of PKC or phosphatase activity had no effect on GLT-1-mediated activity in these transfected cell lines. To determine if GLT-1 regulation by PKC is cell-specific, HeLa cells, which endogenously express the EAAC1 subtype of transporter, were stably transfected with GLT-1. Although EAAC1-mediated activity was increased by activation of PKC, we found no evidence for regulation of GLT-1. Despite the present findings, GLT-1 activity may be regulated by PKC under certain conditions.
glutamate transporters. There is evidence that GLAST-mediated transport can be rapidly down-regulated by activation of protein kinase C (PKC) and subsequent phosphorylation of the transporter (Conradt and Stoffel, 1997). Recent studies suggest that EAAC1-mediated transport is increased by activation of PKC or decreased by an inhibitor of phosphatidylinositol 3-kinase (Davis et al., 1998). These effects on activity are correlated with altered cell surface expression and are consistent with regulation of activity through trafficking to and from the cell surface. There is also evidence that GLT-1 may be rapidly regulated by activation of PKC (Casado et al., 1993; Ganel and Crosson, 1998). In the earlier study, PKC activation increased glutamate transport; in the later study, PKC activation decreased transport activity by increasing the $K_m$ value.

The goal of the present study was to examine the pharmacological properties of the rat homolog, GLT-1, and the possible mechanisms of rapid regulation by PKC. To accomplish these goals, GLT-1 cDNA was stably transfected into cell lines that express low levels of endogenous glutamate transport activity. In two separate cell lines, the pharmacological properties of GLT-1 parallel those observed in crude cortical synaptosomes. These two cell lines were used to study the regulation of GLT-1 by PKC. In both cell lines, glutamate transport activity was unaffected by activation of PKC. Western blotting and analyses of transport activity suggest that the previously reported regulation of GLT-1 may be attributed to regulation of endogenously expressed EAAC1 in the cell lines used in earlier studies.

**Materials.** L-[3H]Glutamate (40–60 Ci/mmol) was purchased from DuPont (Boston, MA). Nonradioactive L-glutamate (Sigma Chemical Co., St. Louis, MO) was used to dilute the specific activity. The sources for all the EAA analogs are listed in Table 1. MCB 3901 (a Syrian hamster adenovirus type-12-induced tumor, catalog no. CRL-9595), L-M (TK-) (a subline of 5-bromo-2-deoxyuridine-resistant strain of the L-M mouse fibroblast cell line, catalog no. CCL-13), and HeLa (epithelioid carcinoma, catalog no. CCL-2) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The GLT-1 cDNA in pBluescript SK- was a generous gift from Dr. Baruch Nunner (Hebrew University, Jerusalem, Israel). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO). Forskolin, bisindolylmaleimide II (Bis), okadaic acid (Procentrum concavum), and A23187-free acid (streptomyces chartreusensis) were purchased from Calbiochem (La Jolla, CA).

**Subcloning, Stable Transfection, and Maintenance of Cell Lines.** L-M (TK-) and MCB cell lines were maintained in Dulbecco's modified Eagle's medium (catalog no. 11960–051, no added glutamine; Gibco BRL, Gaithersburg, MD), 10% heat-inactivated defined fetal bovine serum (catalog no. SH30070.03; Hyclone, Logan, UT), and 1% penicillin (100 U/ml)/streptomycin (100 μg/ml; catalog no. 15140–122; Gibco BRL) in a 7% CO$_2$, 37°C incubator. HeLa cells were maintained in the same media supplemented with 2 mM L-glutamine, and at 5% CO$_2$.

The GLT-1 cDNA was subcloned into pRC/CMV (Invitrogen) using Xhol and XbaI restriction sites. After cesium chloride purification, the cDNA was stably transfected into each cell line using calcium phosphate-DNA precipitation as described previously (Ausubel et al., 1995). 10 μg of pRC/CMV/GLT-1 DNA per 10-cm dish was used. Briefly, cells were incubated with precipitate in 7% CO$_2$ at 37°C for 8 h, then medium containing precipitate was removed, cells were washed twice with 1× PBS and fed with normal tissue culture medium. After incubation for 24 h, increasing concentrations of genetin (catalog no. 11811–031, G418, Gibco BRL) were added to transfected and control cells. Cell culture medium containing G418 was exchanged every 3 to 4 days. After approximately 2 to 3 weeks of selection, several apparently clonal colonies were observed in plates containing cells that had been transfected and maintained with 0.5 mg/ml G418. No untransfected cells survived at this concentration of G418. Several colonies were picked using sterile filter paper circles (6 mm diameter) soaked in trypsin. These colonies were maintained in cell culture medium containing G418. Shortly after expansion to 10-cm dishes, several colonies were frozen and maintained in liquid nitrogen. All experiments were performed with cell lines that had been passaged fewer than 30 times. During this time, there was no apparent trend in transporter expression measured by Western blot or activity.

**Measurement of Na$^+$-Dependent Glutamate Transport Activity.** Transport assays were performed as described previously (Davis et al., 1998). In a 37°C water bath, cultures (plated in 12-well dishes) were prerinsed two times with 1 ml of prerawmed sodium- or choline-containing buffer (Tris base, 5 mM; HEPES, 10 mM; NaCl or choline chloride, 140 mM; KCl, 2.5 mM; CaCl$_2$, 1.2 mM; MgCl$_2$, 1.2 mM; K$_2$HPO$_4$, 1.2 mM; and dextrose, 10 mM). Uptake was initiated by the addition of 1 ml of prerawmed Na$^+$- or choline-containing buffer, which contained L-[3H]glutamate with or without EAA analogs. After 5 min, cultures were rinsed three times with 1 ml of ice-cold choline buffer to stop transport activity. Cells were dissolved in 1 ml of 0.1 M NaOH and a 500 μl aliquot was used to quantitate radioactivity by scintillation spectrometry. Protein was also measured in an aliquot of solubilized cells using a commercial Bradford kit (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA). Na$^+$-dependent transport activity was calculated as the difference in accumulated radioactivity in the Na$^+$-containing and choline-containing buffer. In these assays, less than 10% of the substrate was accumulated and transport was measured under conditions of initial velocity (transport was linear with time beyond 5 min).

**Western Analysis.** After washing twice with ice-cold PBS, cells were harvested using 1 ml per 10 cm dish of Na$^+$-HEPES buffer (20 mM, pH 7.5), containing MgCl$_2$ (0.4 mM), EDTA (0.2 mM), phenyl-
methylsulfonyl fluoride (20 μM), leupeptin (2 μg/ml), and aprotonin (0.22 μg/ml). After sonication and centrifugation at 14,000 RPM in a microcentrifuge for 10 min, an aliquot of the supernatant was used for analysis of protein content and an aliquot was diluted 1:2 in 0.005% bromphenol blue, and 50 mM Tris-Cl, pH 7.0). The samples were frozen at −20°C. Cell culture samples or control brain specimens, prepared as described previously (Schlag et al., 1998), were resolved using 10% polyacrylamide gel electrophoresis. After transfer to polyvinylidine fluoride membranes (Immobilon P, Millipore, Bedford, MA), immunoblots were probed with anti-GLT-1, anti-GLAST, anti-EAAC1, or anti-EAAT4 antibodies (Furuta et al., 1997).

Selection and Characterization of Cell Lines Stably Transfected with GLT-1. One problem with choosing cell lines for stable expression of glutamate transporters is a high level of endogenous activity observed in several of the cell types regularly used for stable transfection, including CHO, COS, or HEK293 (M.B.R., unpublished observations). MCB (Desai et al., 1995), and L-M (TK−) (J.D.R., unpublished observations) express undetectable to low levels of glutamate transport and HeLa cells were previously used for transient transfection of GLT-1 (Pines et al., 1992; Casado et al., 1993).

To examine endogenous expression of transporter subtypes in these cell lines, transporter specific antibodies were used for Western analysis with cortical or cerebellar tissue as a positive control (Fig. 1). A strong immunoreactive band for GLT-1 was observed with a small amount of cortical membrane homogenate, but no GLT-1 immunoreactivity was observed when 80 times more cell line protein was analyzed. Similarly, except for HeLa cells, no EAAT4 or GLAST immunoreactivity was observed in these cell lines; weak immunoreactive bands for these transporters were observed in HeLa cells. No EAAC1 immunoreactivity was observed in L-M (TK−) cells, an extremely faint immunoreactive band was consistently observed in MCB cells (at approximately 64% selectivity comparing cortical and cerebellar tissue).

Curve Fitting and Statistical Analysis. All values reported are the mean ± S.E.M. of at least three independent experiments. Except where noted, IC_{50} values were obtained by fitting experimental data to a theoretical curve with a Hill coefficient of one using One Site Competition method in GraphPad Prism software (GraphPad Prism, version 2.0, GraphPad Software, Inc., San Diego, CA). When two groups were compared, a Student’s unpaired t test was used to compare the values. Multiple groups were compared by ANOVA with post hoc analysis. A p < .05 was considered significant.

Results

Selection and Characterization of Cell Lines Stably Transfected with GLT-1. Table 1 summarizes the IC_{50} values obtained by fitting experimental data to a theoretical curve with a Hill coefficient of one using One Site Competition method in GraphPad Prism software (GraphPad Prism, version 2.0, GraphPad Software, Inc., San Diego, CA). When two groups were compared, a Student’s unpaired t test was used to compare the values. Multiple groups were compared by ANOVA with post hoc analysis. A p < .05 was considered significant.

Table 1: Comparison of potency of EAA analogs for inhibition of GLT-1-mediated transport activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (μM)</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td>L-cysteate (S)</td>
<td>24.7 ± 16</td>
<td>14.7 ± 2.1</td>
</tr>
<tr>
<td>L-anti-endo-3,4-methanopyrrolidine dicarboxylate (T)</td>
<td>12.8 ± 1.0</td>
<td>32.5 ± 14.6</td>
</tr>
<tr>
<td>L-trans-PDC (T)</td>
<td>16.2 ± 5.8</td>
<td>10.2 ± 0.8</td>
</tr>
<tr>
<td>d-aspartate (S)</td>
<td>29.8 ± 2.2</td>
<td>11.9 ± 0.9</td>
</tr>
<tr>
<td>L-aspartate (S)</td>
<td>25.8 ± 2.9</td>
<td>18.3 ± 4.7</td>
</tr>
<tr>
<td>L-2-amino-3-phosphonopropionate (T)</td>
<td>20.7 ± 6.0</td>
<td>16.7 ± 1.4</td>
</tr>
<tr>
<td>x-methyl-D-aspartate (T)</td>
<td>1432 ± 347</td>
<td>234 ± 359</td>
</tr>
<tr>
<td>(RS)-α-amino-3-hydroxy-5-methyl-isoaxazole-4-propanoate (T)</td>
<td>324 ± 32</td>
<td>378 ± 42</td>
</tr>
<tr>
<td>β-N-oxyl-α,β-diaminopropionate (T)</td>
<td>14 ± 5% at 1 mM</td>
<td>8 ± 3% at 1 mM</td>
</tr>
<tr>
<td>dihydrokainate (G)</td>
<td>50.0 ± 20.0</td>
<td>31.6 ± 6.5</td>
</tr>
<tr>
<td>(2S, 1'S, 2'S)-2-(carboxycyclopropyl) glycine (T)</td>
<td>19 ± 4% at 1 mM</td>
<td>2 ± 5% at 1 mM</td>
</tr>
<tr>
<td>α-methyl-α,β-glutamate (T)</td>
<td>3 ± 3% at 1 mM</td>
<td>15 ± 14% at 1 mM</td>
</tr>
<tr>
<td>a-amino adipic acid (T)</td>
<td>3315 ± 104</td>
<td>2434 ± 486</td>
</tr>
<tr>
<td>L-homocysteate (S)</td>
<td>53.4 ± 3.4</td>
<td>70.5 ± 19</td>
</tr>
<tr>
<td>L-quisqualate (T)</td>
<td>1565 ± 392</td>
<td>1970 ± 216</td>
</tr>
</tbody>
</table>

The Na^+ dependent transport of L-[3H]-glutamate (0.5 μM) was measured in the absence and presence of increasing concentrations of inhibitor. All data are mean ± S.E.M. values of at least three independent experiments. IC_{50} values were obtained by fitting the data to one site using GraphPad Prism software. The numbers that precede the name of each compound were used to identify individual points on the graphs correlating the potencies of each compound for inhibition of GLT-1-mediated transport and inhibition of transport activity in cortical or cerebellar synaptosomes (Fig. 5).

a Data from Robinson et al., 1993.

b Data from Robinson et al., 1991.
transfected L-M (TK-) and MCB cells. After transfection, G418-resistant MCB and L-M (TK-) clones were screened for Na\(^+\)-dependent glutamate transport activity and tested for GLT-1 expression by Western blotting (data not shown). Clones were categorized into low, medium, and high levels of expression. The transfected L-M (TK-) cells chosen for further study are referred to as LM-GLT1–8 (Figs. 1 and 2). Compared with other clones identified, LM-GLT1–8 expressed moderate levels of transport activity and GLT-1 immunoreactivity, but still expressed nearly 60-fold higher levels of Na\(^+\)-dependent transport activity than untransfected control cells. The MCB clone chosen for further study (referred to as MCB-GLT1-6) expressed moderate levels of GLT-1 protein compared with other MCB clones. In this cell line, Na\(^+\)-dependent transport activity was 4- to 5-fold that observed in untransfected MCB cells (Figs. 1 and 2). In both cell lines, the Na\(^+\)-independent accumulation of L-[\(^3\)H]-glutamate was unaffected by transfection (-Na\(^+\), Fig. 2).

Kinetic and Pharmacological Properties of L-[\(^3\)H]-Glutamate Transport Activity in Cell Lines Stably Transfected with GLT-1. The concentration dependence of Na\(^+\)-dependent L-[\(^3\)H]-glutamate transport activity was examined in both LM-GLT1–8 and MCB-GLT1–6. Between the concentrations of 1 and 1000 \(\mu\)M L-glutamate, transport activity was consistent with a single saturable process with \(K_m\) values of 37 \(\pm\) 4 \(\mu\)M \((n = 4)\) in LM-GLT1–8 cells and 18.6 \(\pm\) 0.3 \(\mu\)M \((n = 3)\) in MCB-GLT1–6 cells (Fig. 3). The \(V_{max}\) values were 3.1 \(\pm\) 0.4 nmol/mg protein per min in LM-GLT1–8 cells and 2.7 \(\pm\) 0.3 nmol/mg protein per min in MCB-GLT1–6 cells, indicating that both cell lines express a comparable capacity for transport. These capacities for transport are comparable to those observed in cortical, hippocampal, or striatal crude synaptosomes (Robinson et al., 1991).

Several EAA analogs were tested for inhibition of transport activity in both cell lines and were compared to our previously published data for inhibition of transport activity in crude synaptosomes (Table 1). All of the compounds tested inhibited transport activity with comparable potencies (within 2.5-fold) in both LM-GLT1–8 and MCB-GLT1–6 cell lines. In both cell lines, transport activity was inhibited by many of the prototypic inhibitors of Na\(^+\)-dependent glutamate trans-
port including D-aspartate, L-cysteate, L-trans-pyrrolidine-2,4-dicarboxylate (L-trans-PDC; Fig. 4A), and DL-threo-hydroxyaspartate (Table 1, Part A). These compounds have less than 3-fold selectivity for inhibition of transport in either cortex or cerebellum and were 5- to 10-fold more potent as inhibitors of transport in cortical tissue than in GLT-1 transfected cell lines.

In earlier studies with synaptosomes, we had observed inhibition of transport activity by some compounds that also interact with EAA receptors (Robinson et al., 1993). In both LM-GLT1–8 and MCB-GLT1–6 cells we observed essentially no inhibition by N-methyl-D-aspartate. In contrast, the ionotropic receptor agonist, (RS)-α-amino-3-hydroxy-5-methylisoxazole-4-propionate (Table 1, Part A). These compounds have less than 3-fold selectivity for inhibition of transport in either cortex or cerebellum and were 5- to 10-fold more potent as inhibitors of transport in cortical tissue than in GLT-1 transfected cell lines.

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Fig. 3. Eadie-Hofstee plot of the concentration dependence of Na\(^+\)-dependent L-[\(^3\)H]-glutamate transport in LM-GLT1–8 (A) and MCB-GLT1–6 (B). Inset: Saturation isotherm of the same data (Michaelis-Menten). Data are the mean ± S.E.M. values of at least three independent observations, each performed in triplicate. For LM-GLT1–8 cells, the \(V_{\max}\) value was 3.1 ± 0.4 nmol/mg protein per min and the \(K_m\) value was 37 ± 4 \(\mu\)M. For MCB-GLT1–6 cells, the \(V_{\max}\) value was 2.7 ± 0.3 nmol/mg protein per min and the \(K_m\) value was 18.6 ± 0.3 \(\mu\)M.

Fig. 4. Sensitivity of GLT-1-mediated transport to inhibition by L-trans-PDC and DHK. Na\(^+\)-dependent L-[\(^3\)H]-glutamate transport was measured in the absence and presence of increasing concentrations of inhibitors in MCB-GLT1–6 (■) and LM-GLT1–8 (○). Data are mean ± S.E.M. values of three independent observations, each performed in triplicate. The solid lines represent the theoretical curves with Hill coefficient of 1. IC\(_{50}\) values are presented in Table 1. The dotted line represents the previously reported sensitivity of cerebellar glutamate transport to the inhibitor and the dashed line represents the previously reported sensitivity of cortical glutamate transport to the inhibitor (Robinson et al., 1991; 1993).

To determine if the pharmacology of GLT-1 correlates with that observed in cortex, inhibitors selective for either cortical or cerebellar transport were tested (Table 1, Part B). DHK, the only forebrain (hippocampus, cortex, striatum, and midbrain)-selective inhibitor available, blocked transport in both MCB-GLT1–6 and LM-GLT1–8 with IC\(_{50}\) values that were within 2-fold of those observed in cortical synaptosomes (Fig. 4B). This contrasts with cerebellar activity, which is insensitive to DHK. Four compounds with greater than 10-fold selectivity for inhibition of cerebellar transport are available (Table 1, Part B). These compounds include: L-AAD, β-N-oxalyl-L-α, β-diaminopropionate, (2S, 1'S, 2'S)-2-(carboxycyclopropyl) glycine, and α-methyl-DL-glutamate. L-AAD blocked GLT-1-mediated transport with an IC\(_{50}\) value of 3.5 to 3.5 mM. This potency is approximately 4-fold lower than the IC\(_{50}\) value for inhibition of forebrain (cortex, hippocampus, striatum, and midbrain) transport (Robinson et al., review, see Schoepp et al., 1990). Because L-AP3 also inhibits EAAC1-mediated transport activity (Dowd et al., 1996), L-AP3 should be used with caution as an mGluR antagonist.
In cortical tissue, data for inhibition of transport by three compounds (kainate, 
1-homocysteate, and quisqualate) was best fit to two sites with dramatically different affinities (Table 1, Part C). With all three compounds, there was a predominant component that represented approximately 70 to 90% of the sites. The affinities for inhibition of GLT-1 were comparable to those observed for the predominant component in cortex. For example, GLT-1-mediated transport was inhibited by kainate with IC\textsubscript{50} values (50–70 \textmu M) similar to the predominant component in cortical synaptosomes (72 \textmu M).

Given that the patterns of sensitivity of GLT-1 to inhibition by several compounds were similar to that observed in cortex, the pharmacological properties of GLT-1 were compared to those observed in both cerebellar and cortical synaptosomal preparations. To facilitate this comparison, the Log IC\textsubscript{50} values for inhibition of GLT-1-mediated transport were plotted against the Log IC\textsubscript{50} values for inhibition of cortical or cerebellar synaptosomal transport activity (Fig. 5). If one assumes that the compounds interact through a competitive interaction and use the Cheng and Prusoff equation to convert IC\textsubscript{50} values to \( K_i \) values (for original reference, see Dowd et al., 1996), the graphs are essentially identical (not shown). These graphs reveal a significant correlation between the potencies of compounds for inhibition of GLT-1-mediated transport and cortical synaptosomal transport. The slope of the line drawn through this data is 1.000 and the x-intercept is 0.526. The slope of 1 is consistent with a parallel shift in the potencies of the compounds and the x-intercept of 0.526 would indicate that compounds are, on average, 3-fold less potent as inhibitors of GLT-1-mediated transport than of cortical synaptosomal transport.

**Effects of Over-Expression of Glutamate Transporters in Cell Lines.** One approach to limiting the impact of endogenous glutamate transporter expression in cell lines is to express higher levels of transfected transporter relative to that observed in untransfected cells (Matthews et al., 1997). In our initial studies, we decided to attempt a similar approach with an MCB clone (MCB-GLT-12) that expressed much higher levels of GLT-1 protein than MCB-GLT1–6 (Fig. 1). In this cell line, the \( V_{\text{max}} \) value for \( \text{L-[3H]glutamate} \) uptake was 24 ± 5 nmol/mg protein per min (data not shown, \( n = 4 \)), which was almost 10-fold higher than that observed in MCB-GLT1–6. In this cell line, the \( K_m \) value for glutamate transport was four times higher than that observed in MCB-GLT1–6 even though less than 10% of the glutamate was transported from the extracellular space (\( K_m = 78 \pm 30 \text{ \mu M} \)). Similar shifts in the potencies of compounds cleared by transporters have been observed in brain slices and cells in culture (for a recent discussion, see Speliotes et al., 1994), and have been attributed to the rapid clearance of glutamate from the local environment near the transporters. This could explain the lower apparent affinity of the transporter for glutamate, but the pharmacological properties of transport were also affected by this high level of expression. The sensitivity of transport to DHK was much lower in MCB–GLT–12 (IC\textsubscript{50} = 588 ± 89 \textmu M) than in MCB-GLT1–6 (IC\textsubscript{50} = 32 ± 7 \textmu M) as was the sensitivity to inhibition by \( L\text{-trans-PDC} \) (112 ± 42 \textmu M, \( n = 3 \)), kainate (1140 ± 220 \textmu M, \( n = 3 \)), and \( L\text{-anti-endo-3,4-methanopyrrolidine dicarboxylate} \) (108 ± 9 \textmu M, \( n = 3 \)). To rule out the possibility that confluent MCB-GLT1–12 cells express higher levels of EAAC1, GLAST, or EAAT4, protein sample were harvested from confluent cultures. We found no evidence for higher levels of any of these proteins in confluent MCB-GLT1–12 cells than in confluent MCB-GLT1–6 (data not shown, \( n \geq 2 \) independent experiments). Although we cannot rule out the possibility that confluence induces expression of EAAT5 or an uncloned transporter, the observation that all of these inhibition data conform to theoretical curves with a Hill slope of 1 suggests that a single population of sites mediates activity in these confluent cultures. To test
the possibility that high expression might be influencing the pharmacological properties of GLT-1 by changing the local environment close to the cells, the sensitivity of transport to DHK was examined using MCB-GLT1–12 cells grown to approximately 30% confluence. Under these conditions, the IC50 value for inhibition by DHK was 148 μM (n = 2), a value which approaches that observed in 80 to 90% confluent LM-GLT1–8 and MCB-GLT1–6 cells. Thus, it appears that high levels of activity can influence the pharmacological properties.

To determine if this phenomenon might influence the results of the present study, MCB-GLT1–6 cells were grown to less than 40% confluence and the sensitivity to DHK was assessed. Under these conditions, the IC50 value for DHK was indistinguishable from that observed at 80 to 90% confluence (IC50 = 33 μM, n = 2). This suggests that the pharmacological properties of MCB-GLT1–6 are not likely to be affected by confluence of the cells. This also suggests that the phenomenon observed in MCB-GLT1–12 is related to the high levels of GLT-1 expression rather than a generalized change in MCB cells that occurs when they are grown to high confluence.

Effects of Modulation of PKC on GLT-1-Mediated Transport. Using HeLa cells, Casado and her colleagues published a report that suggested that GLT-1-mediated transport could be rapidly increased by activation of PKC (Casado et al., 1993). Because this type of rapid regulation could have significant implications for the control of extracellular EAs, we pursued this regulation of GLT-1 in both stably transfected cell lines. In both cell lines, preincubation with the phorbol ester (PMA) for 30 min had no effect on transport activity (Fig. 6A, mean of at least nine independent observations). As a positive control, each of the stock solutions of PMA was tested for activation of endogenous EAAC1-mediated transport in C6 glioma cells. All stocks used caused an increase in transport activity (data not shown, see Davis et al., 1998 for description of assay). Higher concentrations of PMA (up to 1 μM) and longer incubations (up to 1 h) were also tested and neither caused an increase in GLT-1-mediated transport activity in transfected cells (data not shown, n = 2).

The absence of an effect of PMA could be a consequence of endogenous activation of PKC in these cell lines and/or high levels of protein phosphatase activity which could theoretically exceed the capacity for phosphorylation by PKC. To address these possibilities, the effects of the PKC inhibitor, Bis, on transport activity was examined. At concentrations that blocked the effects of PMA in C6 glioma and using stock solutions that were tested in parallel (data not shown, see Davis et al., 1998 for description of assay), preincubation with 10 μM Bis for 30 min had no effect on GLT-1-mediated transport activity (Fig. 6A). To block phosphatase activity in these cell lines, okadaic acid, which blocks both protein phosphatase 1 and 2A at 1 μM, was tested (Cohen et al., 1990). Preincubation with okadaic acid (1 μM) had no effect on transport activity, nor did the combination of okadaic acid and phorbol ester (Fig. 6B). Our observations suggest that neither endogenous activity of PKC nor high protein phosphatase activity can explain the lack of an effect of PMA on GLT-1-mediated transport. In an earlier study (Casado et al., 1991), it was reported that the Ca2+ ionophore A23187 potentiates the effects of PMA. We found that A23187 by itself or in combination with PMA had no effect on transport activity (Fig. 5B). Together, these data suggest that GLT-1 is not activated by PKC. We also examined the effects of activation of protein kinase A with forskolin and found no change in transport activity (data not shown).

HeLa cells were used in the earlier study that suggested that GLT-1 could be regulated by activation of PKC (Casado et al., 1993). To examine the possibility that regulation of GLT-1 by PKC is cell-specific, HeLa cells were stably transfected with GLT-1. Two clones, HeLa-GLT1–7 and HeLa-GLT1–14, that express moderate levels of GLT-1 immunoreactivity (Fig. 7A and B) were isolated. In both of these cell lines, the expression of EAAC1 was also increased (Fig. 7, C and D), but the expression of GLAST and EAAT4 was not affected by stable transfection of GLT-1 (data not shown, n =

![Fig. 6. Effect of modulation of PKC on GLT-1-mediated transport in LM-GLT1–8 and MCB-GLT1–6 cells. A, cells were pretreated with vehicle dimethyl sulfoxide (DMSO), 100 nM PMA, or 10 μM Bis for 30 min before measurement of Na+-dependent glutamate (0.5 μM) transport. Data are the mean ± S.E.M. of at least three independent observations with PMA and at least three independent observations with Bis. There was no significant effect of either treatment on Na+-dependent glutamate transport activity. B, cells were pretreated with either the phosphatase inhibitor okadaic acid (1 μM) or the Ca2+ ionophore A23187 (100 nM) alone and in combination with PMA (100 nM) for 30 min. Data are the mean ± S.E.M. of at least three independent observations. There was no significant effect of any of the treatments.](https://jpet.aspetjournals.org/doi/10.1124/jpet.117.246700)
In the present study, three different cell lines were stably transfected with the cDNA for the GLT-1 subtype of glutamate transporter. Stably transfected cell lines have been used as ideal model systems to study the function, pharmacology, and post-translational regulation of receptors, channels, and other cell surface proteins. In most cases, the corresponding cDNA is transfected into a cell line that does not endogenously express the protein itself or functional homologs. Choosing a cell line to stably express glutamate transporters presents a challenge because many cells endogenously express these transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of trans- 

The effects of PMA (100 nM for 30 min) were examined in both untransfected and transfected cells. In transfected cells, the absolute increase in transport activity was greater than that observed in untransfected cells (in untransfected cells [2.5 ± 0.4-fold increase, p = .018], it seemed possible that regulation of EAAC1 by PKC might be sufficient to explain the up-regulation of glutamate transport activity by PMA in these transfected cells. Because previous studies (and the present pharmacological analyses) suggest that DHK inhibits GLT-1-mediated transport at lower concentrations than are required to inhibit EAAC1-mediated transport, the sensitivity of transport to inhibition by DHK was examined in HeLa-GLT1–7 (Fig. 8B). The IC50 value for inhibition of GLT-1 is approximately 40 μM (this study) and the IC50 value for inhibition of EAAC1 is approximately 1 mM (see Robinson and Dowd, 1997). In HeLa-GLT1–7 cells, the data for inhibition of transport activity by DHK were best fit to two sites with IC50 values of 16 ± 5 and 1,200 ± 100 μM (n = 3). The percentage of the high-affinity site was 31 ± 4% of the total. Although the observed up-regulation of EAAC1 makes it extremely difficult to directly determine if GLT-1 is activated by PKC, we utilized this differential sensitivity to DHK to indirectly determine if GLT-1 is regulated by PMA. HeLa-GLT1–7 were first preincubated with PMA (100 nM) for 30 min and then transport activity was measured in the absence or presence of 300 μM DHK (Fig. 8C). When transport activity was measured in the presence of DHK, PMA increased activity from 10.7 ± 0.7 to 19 ± 0.6 pmol/mg protein per min. When transport activity was measured in the presence of DHK, PMA increased activity from 6.7 ± 0.3 to 17 ± 0.0 pmol/mg protein per min. In fact, the average PMA-induced increase in transport activity (delta) was not significantly different when activity was measured in the presence or absence of DHK (300 μM). These data suggest that the transport activity mediated by the component with higher sensitivity to inhibition by DHK (presumably GLT-1) was not regulated by PMA.

**Discussion**

In the present study, three different cell lines were stably transfected with the cDNA for the GLT-1 subtype of glutamate transporter. Stably transfected cell lines have been used as ideal model systems to study the function, pharmacology, and post-translational regulation of receptors, channels, and other cell surface proteins. In most cases, the corresponding cDNA is transfected into a cell line that does not endogenously express the protein itself or functional homologs. Choosing a cell line to stably express glutamate transporters presents a challenge because many cells endogenously express these transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes. One approach to overcoming the impact of endogenous expression might be to select a clone that expresses high levels of transporters, presumably to provide glutamate and/or aspartate for metabolic purposes.
endogenous transport in untransfected MCB cells did not significantly affect the properties of GLT-1. Some of the compounds tested in the present study have been tested as inhibitors of GLT-1 in earlier studies. In the original paper that described the cloning and expression of the rat homolog of GLT-1, HeLa cells were used as an expression system and both DHK and l-AAD were reported to potently inhibit transport activity (IC50 values < 8 µM; Pines et al., 1992). Arriza and his colleagues expressed the human homolog of GLT-1 (EAAT2) in COS-7 cells and found that it is blocked by DHK with an IC50 value of 23 µM and is not blocked by 1 mM l-AAD (Arriza et al., 1994). While the present study was underway, Wang and colleagues reported that the rat homolog of GLT-1 expressed in oocytes is inhibited by DHK with an IC50 value of 8 µM and that the IC50 value for l-AAD is greater than 1 mM (Wang et al., 1998). Dunlop and his colleagues reported that the human homolog of GLT-1 (EAAT2) expressed in Madin Darby Canine kidney cells is inhibited by DHK with an IC50 value of 15 µM and that the IC50 value for l-AAD is greater than 1 mM (Dunlop et al., 1998). In the present study, DHK inhibited GLT-1-mediated transport with an IC50 value of approximately 30 to 50 µM and l-AAD inhibited transport with an IC50 value of approximately 2 to 3 mM. Thus, the sensitivity to l-AAD observed in the original study has not been observed by four other groups. It is possible that this difference is because the transporter was solubilized and reconstituted in the original study.

The sensitivity of GLT-1-mediated transport to inhibition by a number of compounds is remarkably similar to that observed in cortical tissue. Because the pharmacologies of the brain glutamate transporters, GLAST, EAAC1, and EAAT4, are different from those of cortex (see introduction), the simplest interpretation of these results is that GLT-1 mediates the bulk of cortical glutamate transport activity. Although the pharmacology of transport in hippocampus, striatum, and midbrain has been examined with only a limited number of compounds, the pharmacological properties of GLT-1 are generally consistent with transport in these brain regions, suggesting that GLT-1 mediates the bulk of transport activity in forebrain and midbrain. Although one cannot rule out the possibility that an uncloned transporter mediates activity in these brain regions, GLT-1 gene deletion and knock-down studies also support this conclusion. Infusion (ICV) of antisense oligonucleotides specific for GLT-1 decreases protein expression by 60% and causes a 50% decrease in brain transport activity (Rothstein et al., 1996). Genetic deletion of GLT-1 (Tanaka et al., 1997) and pathologic loss of GLT-1 in amyotrophic lateral sclerosis (Rothstein et al., 1995) are also associated with a significant loss (up to 90%) of

Fig. 8. Analyses of Na+-dependent l-[3H]-glutamate transport activity in HeLa cells stably transfected with GLT-1 cDNA. A, Eadie-Hofstee plot of the concentration dependence of Na+-dependent l-[3H]-glutamate transport in untransfected HeLa (O) and HeLa-GLT1–7 cells (○). Data are the mean ± S.E.M. values of at least three independent observations, each performed in triplicate. For untransfected HeLa cells, the Vmax value was 212 ± 74 pmol/mg protein per min and the Km value was 37 ± 20 µM. For HeLa-GLT1–7 cells, the Vmax value was 1300 ± 230 pmol/mg protein per min and the Km value was 16 ± 1 µM. B, sensitivity of glutamate transport activity (0.5 µM) to inhibition by DHK in HeLa-GLT1–7. Transport activity was measured in the absence and presence of increasing concentrations of DHK. Data are mean ± S.E.M. values of three independent observations, each performed in triplicate. Data were fit to two sites using GraphPad Prism. The equation used to fit two sites was constrained such that the sum of the fraction of high-affinity sites and the fraction of low-affinity sites equaled 1. The mean IC50 values were 16 ± 5 and 1,200 ± 100 µM and the mean fraction of the high-affinity component was 0.31 ± 0.04. The solid line represents the theoretical curve for the two-site fit of these data and the dashed line represents a theoretical curve for a one-site fit of these data (IC50 value = 378 µM). C, effect of modulation of PMA on glutamate transport activity in HeLa-GLT1–7. Cells were pretreated with vehicle (DMSO) or 100 nM PMA for 30 min before measurement of Na+-dependent glutamate (0.5 µM) transport activity. Transport activity was measured in the absence or presence of 300 µM DHK. Delta represents the difference in activity measured in cells treated with DMSO or PKC. Data are the mean ± S.E.M. of three independent observations each performed in triplicate. Although PMA caused a significant increase in transport activity (P < 0.01), the PMA-induced increase in transport activity was not affected by DHK.
transport activity in brain tissues. One limitation of these studies is that the neurodegeneration that accompanies these decreases in GLT-1 may lead to an overestimation of the contribution of GLT-1 to transporter activity.

If GLT-1 is indeed the predominant cortical (forebrain) transporter, it raises the possibility that nerve terminals do not accumulate significant levels of glutamate after release, but there are early lesioning studies that originally suggested that there was significant transport into neurons. In these studies, several groups demonstrated that lesioning of afferents decreases transport activity in the target area (for review, see Fagg and Foster, 1983). Although the original interpretation of these data was that transporters were localized to nerve terminals, more recent studies have demonstrated that these lesions result in decreased expression of the glial transporters (Ginsberg et al., 1995; Levy et al., 1995). This suggests that neurons participate in the regulation of expression of the glial transporters in vivo. In fact, in vitro studies have demonstrated that GLT-1 expression in astrocytes is dependent on the presence of neurons (Gegelashvili et al., 1997; Swanson et al., 1997; Schlag et al., 1998). Thus, some of the early observations that suggested that neurons may be important for the clearance of glutamate can be explained by glial transport.

Still, there are observations that cannot be easily explained. First, GLT-1 protein levels in cerebellar synaptosomes are comparable to that observed in forebrain synaptosomes (Robinson, 1998). Yet, no DHK sensitivity is observed in cerebellar synaptosomal membrane preparations (for review, see Robinson and Dowd, 1997). Second, transport in neuron-enriched cultures, which express only low levels of GLT-1 immunoreactivity, has a pharmacology that is comparable to that observed in cortical synaptosomes (Wang et al., 1998). Although this suggests that there may be another transporter with properties similar to GLT-1, the authors did not exclude the possibility that this low level of GLT-1 expression may explain the observed pharmacology.

In an earlier report, Casado et al. (1993) concluded that GLT-1 can be regulated by activation of PKC. In the present study, we found no evidence for regulation of GLT-1-mediated transport activity by PKC. Activation of PKC, inhibition of PKC, and activation of PKC in the presence of a phosphatase inhibitor had no detectable effect in two independent cell lines (LM-GLT1–8 and MCB-GLT1–6). In this earlier report, C6 glioma cells were used to demonstrate phosphorylation of an immunoprecipitable band. We have found that C6 glioma cells express EAAC1 but not GLT-1 or the other transporters (Dowd et al., 1996, Davis et al., 1998). Using reverse transcription-polymerase chain reaction another group (Palos et al., 1994) also has concluded that C6 glioma express only EAAC1. Because EAAC1 is rapidly increased in response to vaccinia virus (for review, see Rob- inson and Dowd, 1997), this suggests that extracellular EAs are released from the nerve terminal recycle through the astrocyte rather than through reuptake directly into the nerve terminal. Although there is substantial evidence for post-translational regulation of other transporters and cell surface proteins by PKC (for references, see Davis et al., 1998), we were unable to detect any effect of PKC stimulation on GLT-1 activity.

In conclusion, examination of the properties of the GLT-1 transporter reveals that its pharmacological characteristics are sufficient to explain the majority of activity in crude synaptosomes prepared from cortex and possibly other forebrain regions. Because GLT-1 is generally thought to be expressed by mature astrocytes in vivo (for review, see Robinson and Dowd, 1997), this suggests that extracellular EAs released from the nerve terminal recycle through the astrocyte rather than through reuptake directly into the nerve terminal. Although there is substantial evidence for post-translational regulation of other transporters and cell surface proteins by PKC (for references, see Davis et al., 1998), we were unable to detect any effect of PKC stimulation on GLT-1 activity.

Acknowledgments

We thank Brian Schlag for his help with preparing the transfected cell lines, Dr. Paul Rosenberg for his helpful discussions regarding the explanation for the effects of overexpression of transporters on their kinetic and pharmacological properties, and Dr. Baruch Kan- ner for the GLT-1 cDNA. We also thank Anjali Gupta for her excellent editorial assistance.

References


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