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Comparison of Na^+-Dependent Glutamate Transport Activity in Synaptosomes, C6 Glioma, and *Xenopus* Oocytes Expressing Excitatory Amino Acid Carrier 1 (EAAC1)

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SUMMARY

Several subtypes of sodium-dependent high affinity (SDHA) glutamate transporters have been pharmacologically differentiated in brain tissue. Recently, four distinct cDNAs (EAAC1, GLT1, GLAST, and EAAT4) encoding Na^+-dependent glutamate transporters have been isolated, but the properties of some of these transporters do not fully match the properties of transport observed in brain tissue or astrocyte-enriched cultures. The purpose of the current investigation was to determine whether the pharmacological properties of EAAC1 parallel those observed in cortical or cerebellar synaptosomes, C6 glioma, or primary astrocyte-enriched cultures. EAAC1 cRNA was expressed in *Xenopus* oocytes, an expression system with no detectable endogenous Na^+-dependent glutamate transport activity. EAAC1-mediated glutamate transport was >98% Na^+ dependent, and the transport was saturable and consistent with a single site. Glutamate transport activates in EAAC1-injected oocytes and C6 glioma have similar K_m values for glutamate (K_m = 15–24 μM) and Na^+ (apparent K_m = 35–50 mM), and these values markedly differ from those observed in rat synaptosomes (glutamate, K_m = 1–5 μM; Na^+, K_m = 13–20 mM). Several excitatory amino acid analogues were tested as inhibitors of L-[^{3}H]glutamate transport in oocytes expressing EAAC1 cRNA. The potencies of several compounds for inhibition of EAAC1-mediated transport differed from those previously observed in cerebellar synaptosomes and astrocyte-enriched cultures. Although EAAC1-mediated transport and cortical synaptosomal transport have similar pharmacological profiles, five excitatory amino acid analogues were ≥3-fold more potent as inhibitors of transport into cortical synaptosomes than of transport into EAAC1-injected oocytes. For example, L-*trans*-pyrrolidine-2,4-dicarboxylate was ~5-fold more potent in cortical synaptosomes, and dihydrokainate was ~10-fold more potent in cortical synaptosomes than in EAAC1-injected oocytes. In contrast, all of the compounds examined inhibit transport observed in C6 glioma with potencies similar to that observed in oocytes injected with EAAC1 cRNA. Consistent with these data, C6 glioma expressed EAAC1- but not GLT1- and GLAST-like immunoreactivity. Although this immunoreactivity migrated as proteins of slightly different molecular masses in each system, treatment with *N*-glycosidase F shifted all proteins to a molecular mass consistent with that predicted from the cDNA sequence. In cortical synaptosomes, EAAC1-, GLT1-, and GLAST-like immunoreactivities were apparent. These results indicate that (i) EAAC1 but not GLAST or GLT1 transporters are expressed in C6 glioma, (ii) synaptosomes contain a heterogeneous population of transporters, (iii) EAAC1 does not account for the pharmacology previously observed in cortical synaptosomes, and (iv) based on the pharmacology and tissue distribution of EAAC1, GLT1, GLAST, and EAAT4, it appears that there are additional glutamate transporter subtypes.

Glutamate and aspartate are the predominant excitatory neurotransmitters in the mammalian central nervous system (for reviews, see Refs. 1 and 2). Unlike other neurotransmitters, which are removed from the synapse by both enzymatic metabolism and uptake processes, extracellular EAA concentrations are generally thought to be regulated by uptake via SDHA transporters, with no extracellular metabolism (for reviews, see Refs. 1 and 3).

There is pharmacological and molecular biological evidence that SDHA transport of EAAs is mediated by several transporter subtypes. SDHA L-[^{3}H]glutamate transport measured in synaptosomes prepared from forebrain has been

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ABBREVIATIONS: EAA, excitatory amino acid; SDHA, sodium-dependent high affinity; DHK, dihydrokainate; L-AAD, L-α-aminoadipate; L-*trans*-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylate; DL-THA, DL-*threo*-β-hydroxyaspartate; L-CCGIII, (2*S*,1′*S*,2′*R*)-2-(carboxycyclopropyl) glycine; L-CCGIV, (2*S*,1′*R*,2′*S*)-2-(carboxycyclopropyl) glycine; NMDA, *N*-methyl-D-aspartate; L-CCGII, (2*S*,1′*R*,2′*R*)-2-(carboxycyclopropyl) glycine; L-ODAP, β-*N*-oxalyl-L-α,β-diaminopropionic acid; L-CCGI, (2*S*,1′*S*,2′*S*)-2-(carboxycyclopropyl) glycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

pharmacologically differentiated with several EAA analogues from transport measured in synaptosomes prepared from cerebellum (4–6). For example, cerebellar transport is DHK insensitive, and cortical transport is DHK sensitive (IC_{50} = 100 μM). In synaptosomes prepared from cortex and/or cerebellum, data for inhibition of L-[^{3}H]glutamate transport by several compounds are best fit to two sites or two components (6). Based on the heterogeneity of transport between brain regions and the further heterogeneity within each particular brain region, these data suggest that there are at least four subtypes that mediate glutamate transport in synaptosomes. In addition, transport into astrocyte-enriched cultures prepared from cerebellum and cortex can be differentiated from transport into synaptosomes (7), thereby providing evidence for a fifth subtype that mediates transport into astrocyte-enriched cultures. There is additional evidence for subtypes of transport in other brain regions (8–11).

Three distinct cDNAs encoding glutamate transporters were originally isolated: rat GLT1 (12), rabbit EAAC1 (13), and rat GLAST (14, 15). These proteins share 50–55% amino acid sequence identity (for reviews, see Refs. 16 and 17). Human homologues of the GLT1, EAAC1, and GLAST subtypes have also been isolated (18, 19). Fairman *et al.* (20) isolated a cDNA encoding a fourth subtype of glutamate transporter, designated EAAT4, from a human cDNA library.

All four cDNAs confer SDHA glutamate transport when expressed in heterologous expression systems. The pharmacological profile and cellular localization of the GLAST subtype parallel those seen in astrocyte-enriched cultures (7, 15). The human EAAT4 subtype is exclusively localized to cerebellar neurons, and its L-AAD sensitivity resembles that of cerebellar synaptosomes (5, 6, 20). The pharmacological properties of GLT1 and EAAC1, however, are not observed in rat brain synaptosomes or astrocyte-enriched cultures. For example, transport mediated by the GLT1 subtype is potently inhibited by DHK and L-AAD, a pattern of sensitivity unlike that in synaptosomes from six different brain regions or in astrocyte-enriched cultures (5, 7). Transport mediated by the rabbit homologue of EAAC1 is inhibited by L-AAD (K_i = 165 μM) and is essentially DHK insensitive (13). Although the pharmacology of this rabbit transporter agrees with that observed in rat cerebellar synaptosomes, EAAC1-like immunoreactivity is highly enriched in both cerebellum and cortex (21). Therefore, it is not clear whether the properties of EAAC1 parallel the pharmacology and localization predicted from earlier studies. Alternatively, this discrepancy may be due to species differences, as has been observed with serotonin transporters (22).

The goals of the current study were to characterize glutamate transport mediated by the rat homologue of EAAC1 expressed in *Xenopus* oocytes and to compare it with the properties of rat glutamate transport observed in synaptosomes, astrocyte-enriched cultures, and C6 glioma. These data provide direct evidence that the pharmacological properties of EAAC1 do not parallel the properties observed in either synaptosomes or astrocyte-enriched cultures but do parallel the properties of transport observed in C6 glioma. Portions of these findings have appeared in abstract form (23, 24).

Experimental Procedures

Materials. Adult male Sprague-Dawley rats (8–15 weeks old) were obtained from Charles River (Wilmington, MA). *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI). L-[^{3}H]glutamate (specific activity, 56.6 μCi/mmol) was obtained from DuPont (Boston, MA) and diluted with nonradioactive L-glutamate obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase A and *N*-glycosidase F were obtained from Boehringer Mannheim Biochemicals. D-aspartate, L-aspartate, L-cysteine, D-glutamate, L-AAD, kainate, α-methyl-DL-glutamate, L-homocysteate, and 3-aminobenzoic acid ethyl ester (Tricaine) were obtained from Sigma. L-*trans*-PDC, L-(+)-2-amino-3-phosphonopropionic acid, α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid, L-ODAP, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid, L-CCGIII, and L-quisqualate were obtained from Tocris Neuramin (Bristol, UK). DL-THA was obtained from CalBiochem. NMDA and DHK were obtained from Cambridge Research Biochemicals. L-CCGI, L-CCGII, and L-CCGIV were synthesized as previously described (25, 26) and were provided by Drs. K. Shimamoto, M. Ishida, H. Shinozaki, and Y. Ohfune (); enantiomeric purity was >99% as determined by high performance liquid chromatography with an optically active column. All other materials were obtained from Sigma and were of the highest purity available.

Expression of EAAC1 in oocytes. Frogs were anesthetized with 0.17% 3-aminobenzoic acid ethyl ester for 30 min. Ovarian fragments were dissected, rinsed in OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 5 mM HEPES, pH 7.4), and treated with collagenase A (2 mg/mL) for 2 hr. Oocytes were rinsed and stored at 18° in ND-96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 5 mM HEPES, pH 7.4), which was supplemented with 1.8 mM $CaCl_2$, 5% heat-inactivated horse serum (Sigma), and penicillin (100 units/ml)/streptomycin (100 μg/ml). The pBluescript SK$^-$ (Stratagene) plasmid containing the rat cDNA homologue of EAAC1 was a generous gift from Dr. Matthias Hediger (Harvard Medical School, Boston, MA) (accession nos. U39555 and D63772). The cDNA was linearized with *Not*1, and cRNA transcripts were generated using the T7 mMESSAGE mMACHINE (Ambion, Austin, TX). Except where noted, oocytes were injected with 9–12 ng of cRNA (volume, 18.4 nl) or sterile ddH_2O (volume, 18.4 nl) as a control. Injected oocytes were stored at 18° in ND-96, which was supplemented with 1.8 mM $CaCl_2$, 5% horse serum, and penicillin/streptomycin. Transport activity was measured 18–20 hr after injection.

Transport assay in oocytes. Oocytes were incubated for 15 min at room temperature (22°) in individual wells of a 24-well plate in 500 μl of ND-96 containing 1.8 mM $CaCl_2$ and 0.5 μM L-[^{3}H]glutamate. Transport assays were performed in the absence or presence of inhibitor. To assess Na^+ dependence, the uptake was measured in the absence of Na^+ by substituting equimolar choline chloride for the NaCl. Assays were initiated by the addition of three oocytes to each well, and the plates were agitated at 150 rpm on an orbit shaker (Lab-Line, Melrose Park, IL). Uptake assays were terminated by transferring the oocytes through three 1-ml washes of ND-96 supplemented with 1.8 mM $CaCl_2$. L-[^{3}H]glutamate was released from individual oocytes with the use of 500 μl 1% sodium dodecyl sulfate, and the amount of radioactivity was determined by scintillation spectrometry. Sodium-dependent uptake was calculated to be the difference between the amount of radioactivity obtained in the presence of Na^+ and the amount obtained in the choline-containing buffer.

To examine the effects of Na^+ concentration on glutamate transport activity in EAAC1-injected oocytes (0.5 μM glutamate and 10 μM glutamate), the concentration of NaCl was varied while choline chloride was substituted to keep the solution iso-osmotic.

Membrane preparation and transport assay in synaptosomes. Crude synaptosomes were prepared from cortex, and transport was measured as previously described (5, 6). After homogenization in 0.32 M sucrose, crude synaptosomes were prepared and washed in a series of centrifugation steps. SDHA transport of

L-[^{3}H]glutamate was measured in a total volume of 500 μl. After a 3-min incubation, assays were stopped by the addition of ice-cold choline containing buffer. The membranes were filtered with the use of a cell harvester.

Transport assay in C6 glioma. C6 glioma were grown in 12-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and penicillin (100 units/ml)/streptomycin (100 μg/ml) and stored in a 5% CO_2 incubator. Transport assays were performed after the cells reached 80% confluency, as described by Garlin *et al.* (7). After two washes in transport buffer, SDHA transport of L-[^{3}H]glutamate into the cells was measured in a total volume of 1 ml. After a 5-min incubation, assays were stopped by three 1-ml washes with ice-cold choline containing buffer. The cells in each well were then solubilized with 1 ml 0.1 N NaOH, and 500 μl was transferred to 5 ml of Cytoscint (ICN; Aurora, OH). Radioactivity was determined by scintillation spectrometry.

To examine the effects of Na^+ concentration on glutamate transport activity in C6 glioma (0.5 μM glutamate and 100 μM glutamate), the concentration of NaCl was varied while choline chloride was substituted to keep the solution iso-osmotic.

Western blot analyses. Cortical synaptosomes were prepared as described above, except the final resuspension was in 0.32 M sucrose containing protease inhibitors (1 mg/ml soybean trypsin inhibitor, 1 mM phenanthroline, 1 μg/ml leupeptin, 1 mM iodoacetamide, 1 μM pepstatin A, and 250 μM phenylmethylsulfonyl fluoride). Proteins from C6 glioma were prepared by adding 5 ml of Versene (0.02% EDTA in Hanks' buffered saline solution) to a 35-mm tissue culture plate containing a confluent layer of cells. Once the cells lifted from the plate, they were centrifuged at 1500 rpm for 5 min. The media were removed, and the cells were resuspended in 500 μl 0.32 M sucrose containing protease inhibitors. Proteins from oocytes were prepared by homogenizing 10–15 oocytes in 0.32 M sucrose containing protease inhibitors. After protein determinations, cortical synaptosomes, C6 glioma, and oocytes were diluted with denaturing loading buffer and boiled for 5 min. The denatured proteins were then stored at −20° until electrophoresis.

To determine the extent of *N*-linked glycosylation in cortical synaptosomes, C6 glioma, and oocytes, we treated proteins with *N*-glycosidase F (10 units) for 3–14 hr at 37°. Denaturing loading buffer was added to the deglycosylated proteins, and samples were boiled for 5 min. The denatured proteins were then stored at −20° until electrophoresis.

As previously described (21), proteins were electrophoresed on 8% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Bedford, MA). Immunoblots were immersed for 1 hr in blocking solution that contained 0.5% nonfat dry milk, 0.1% Tween-20, and 50 mM Tris-buffered saline. The blots were then incubated for an additional hour in blocking buffer containing the different affinity-purified antibodies. Anti-GLT1, -GLAST, and -EAAC1 antibodies were diluted to 0.034, 0.4, and 0.6 μg/ml, respectively (21). After washing, the blots were incubated for 1 hr with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000 in blocking buffer). The blots were washed again, and the immunoreactive proteins were visualized with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Curve fitting and statistical analysis. Eadie-Hofstee transformations of the concentration-dependence of uptake were fit by linear regression analysis. The concentration dependence for inhibition was examined with only compounds that inhibited transport activity by >50% at 1 mM. For inhibition data that conformed to theoretical curves, IC_{50} values are weighted mean values assuming a theoretical curve with a Hill coefficient of 1. K_i values were calculated from the IC_{50} values with the use of an equation that assumes competitive inhibition (27). Data for most of the inhibitors whose IC_{50} values were transformed into K_i values are consistent with a competitive mechanism of action (5, 6). As is required for this data transformation, these experiments were conducted under conditions of initial velocity, and the concentration of glutamate did not change significantly during the incubation.

For Hill analyses of the Na^+ data, at least six Na^+ concentrations were used to produce 10–90% of maximal transport.

Results

Properties of EAAC1-mediated glutamate transport in oocytes. In the initial studies, oocytes were injected with various amounts of EAAC1 cRNA (1–50 ng). The dependence of transport on the amount of cRNA injected followed an inverted U-shaped curve. The maximum difference between cRNA-injected and water-injected oocytes was observed at ~9–12 ng/oocyte (data not shown; repeated in at least three different experiments). The time dependence of expression of transport activity was also examined at various times (18, 24, 48, and 72 hr) after injection. The maximum difference between cRNA-injected and water-injected oocytes was observed at 18–24 hr after injection (data not shown; repeated in at least three different experiments). In all subsequent experiments, 9–12 ng of cRNA was injected into each oocyte, and transport activity was assessed at 18 hr after injection. Glutamate transport in EAAC1-injected oocytes was ≥10–50-fold greater than glutamate transport in water-injected oocytes (Fig. 1).

The Na^+ and time dependence of L-[^{3}H]glutamate uptake into EAAC1-injected oocytes was examined. At 0.5 μM glutamate, uptake was linear for ≥30 min (Fig. 1A), and at 30 min <10% of the glutamate was transported into the oocytes. Substitution of equimolar choline chloride for the NaCl reduced the uptake to <2 ± 1% of control levels (Fig. 1B). No Na^+-dependent transport of L-[^{3}H]glutamate was observed in oocytes injected with water. The signal observed in oocytes injected with EAAC1 and incubated in the absence of Na^+ was comparable to the signal observed in oocytes injected with water and incubated in the presence of Na^+.

Kinetic analyses of EAAC1-injected oocytes, C6 glioma, and cortical synaptosomes. The concentration dependence of SDHA L-[^{3}H]glutamate transport was examined in EAAC1-injected oocytes (Fig. 1C) and compared with that observed in C6 glioma and with that previously reported in synaptosomes and astrocyte-enriched cultures (Table 1). In both EAAC1-injected oocytes and C6 glioma, transport of L-[^{3}H]glutamate was consistent with a single site (K_m = ~20 μM). These values were 3–20-fold higher than that previously

TABLE 1

Summary of kinetic constants for L-[^{3}H]glutamate transport into synaptosomes, C6 glioma, and EAAC1-injected oocytes

Transport measurements and kinetic constants were obtained as described in Experimental Procedures. Cortical and cerebellar synaptosomal data are from Robinson *et al.* (5, 6). Astrocyte data are from Garlin *et al.* (7). Values are mean ± standard error of at least three observations.

System	K_m for glutamate	Apparent K_m for Na^+	Hill slope for Na^+
	μM	mM	
EAAC1-injected oocytes	15.2 ± 1.1	35.0 ± 1.4	2.5 ± 0.4
C6 glioma	24.3 ± 3.1	51.8 ± 1.4	1.9 ± 0.2
Cortical synaptosomes	5.2	9–20	2.0
Cerebellar synaptosomes	1.9	7–13	2.8
Cortical astrocytes	91	n.d.	n.d.
Cerebellar astrocytes	66	n.d.	n.d.

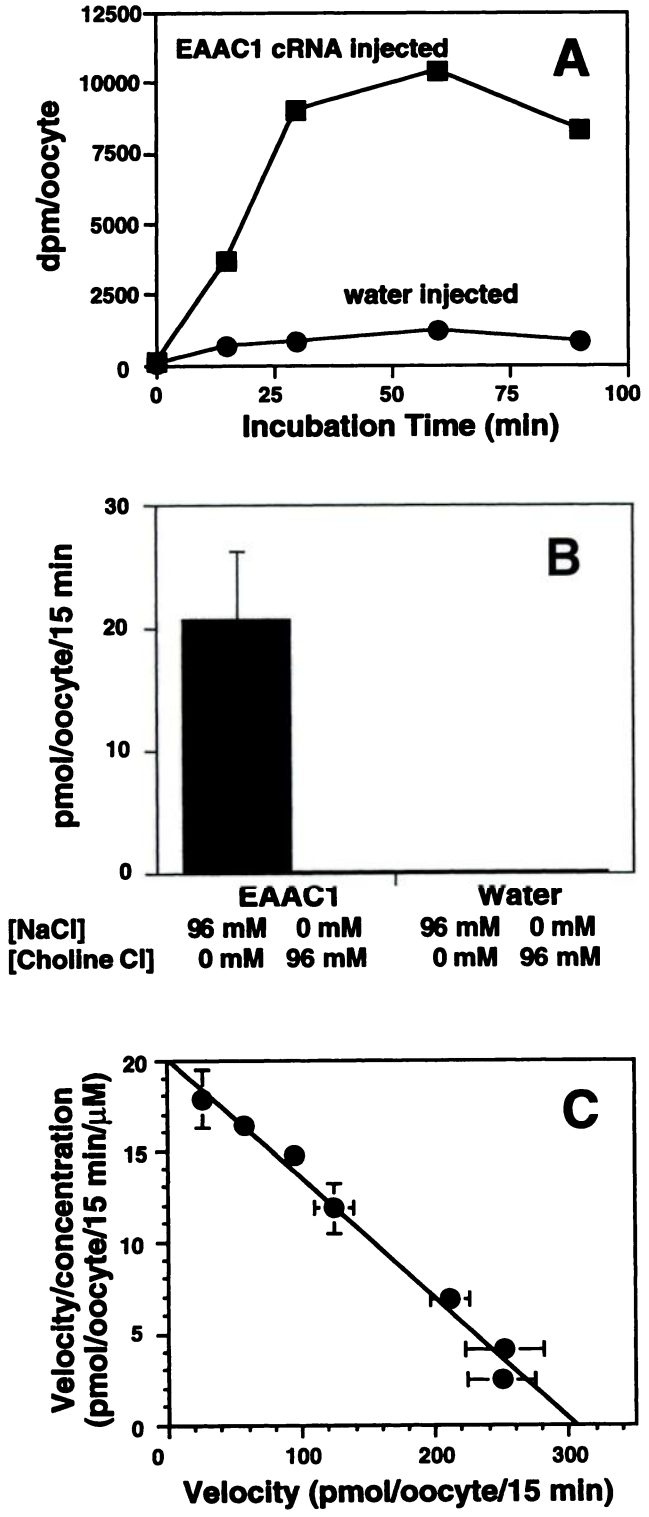

Fig. 1. A, Time course of L-[^{3}H]glutamate transport into *Xenopus* oocytes injected with EAAC1 cRNA. Oocytes were injected with 10 ng (in a volume of 18.4 μl) of *in vitro* transcribed EAAC1 cRNA or ddH_2O. Data are from a representative experiment that was repeated an additional two times. Each data point is the mean of a triplicate determination. B, Sodium dependence of L-[^{3}H]glutamate uptake in EAAC1-injected and water-injected oocytes. To assess Na^+ dependence, the uptake was measured in the absence of Na^+ by substituting equimolar choline chloride for the NaCl. L-[^{3}H]glutamate transport was >98% Na^+ dependent. Data are the mean ± standard error of three independent experiments, and each experiment was performed in triplicate. C, Eadie-Hofstee plot of the concentration dependence of Na^+-dependent L-[^{3}H]glutamate transport in EAAC1-injected oocytes. Data are the mean ± standard error of three independent observations. From this average set of data, the K_m was 15.2 ± 2 μM and the V_{max} was 296 ± 27 pmol/oocyte/15 min.

observed in either cerebellar or cortical synaptosomes and 3–4-fold lower than that previously observed in astrocyte-enriched cultures prepared from either cerebellum or cortex. The capacity (V_{max}) of C6 glioma for L-[^{3}H]glutamate was 430 ± 80 pmol/mg/min.

The effects of altering the external concentration of Na^+ were also examined at different concentrations of L-[^{3}H]glutamate in C6 glioma (0.5 and 100 μM glutamate) and EAAC1-injected oocytes (0.5, 10, and 100 μM glutamate), and these values were compared with those previously reported for uptake into cortical and cerebellar synaptosomes measured at 0.5 μM glutamate (Table 1). Transport activity in cerebellar synaptosomes has the lowest apparent K_m value of ~13 mM (0.5 μM glutamate) (6). Transport into EAAC1-injected oocytes and C6 glioma had higher apparent K_m values (35 ± 2 and 52 ± 3 mM, respectively; 0.5 μM glutamate). The Hill coefficients for transport into EAAC1-injected oocytes, C6 glioma, and cortical synaptosomes were ~2 (Hill slope = 2.5 ± 0.7, 1.9 ± 0.4, and 2, respectively; (0.5 μM glutamate). In C6 glioma, the apparent K_m values for Na^+ increased (0.5 μM glutamate, 52 ± 3 mM; 100 μM glutamate, 34 ± 3 mM) when the glutamate concentration was increased. In EAAC1-injected oocytes, the K_m value for Na^+ was the same in the presence of 0.5 and 10 μM glutamate. Unfortunately, no K_m value for Na^+ was obtained from the EAAC1-injected oocytes in the presence of 100 μM glutamate because the amount of L-[^{3}H]glutamate transported was too low for detection.

Inhibition of EAAC1-mediated glutamate transport by EAA analogues. Twenty-one EAA analogues were tested as inhibitors of L-[^{3}H]glutamate transport in oocytes injected with EAAC1 cRNA (Table 2). Full inhibition curves were generated for compounds that inhibited transport activity by >50% at 1 mM. In addition, some of the compounds that were previously identified as being >10-fold more selective at inhibiting L-[^{3}H]glutamate transport in synaptosomes prepared from either cerebellum or cortex (e.g., L-AAD, DHK, L-ODAP, and α-methyl-DL-glutamate) (6) were tested at a full range of concentrations. Full concentration curves were also examined for compounds whose inhibition of transport into synaptosomes was consistent with two components or sites, including L-CCGII, L-homocysteate, quisqualate, and kainate (6).

D-aspartate, L-aspartate, DLTHA, L-cysteine, L-CCGIII, and L-*trans*-PDC all inhibited the transport of L-[^{3}H]glutamate with K_i values in the range of 1–100 μM. L-CCGIV, L-(+)-2-amino-3-phosphonopropionic acid, NMDA, L-CCGII, and quisqualate were less potent as inhibitors of transport, with K_i values of 100–900 μM. D-glutamate, DHK, L-ODAP, α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid, L-AAD, kainate, α-methyl-DL-glutamate, L-CCGI, (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylic acid, and L-homocysteate were weak inhibitors, with K_i values of ≥900 μM. Table 2 presents the inhibition data, with the most potent inhibitors of EAAC1-mediated transport listed first, followed by the weaker inhibitors.

The potencies of these compounds were initially compared with those previously observed in synaptosomal membranes

TABLE 2
Comparison of potency of EAA analogues for inhibition of EAAC1-mediated glutamate transport

Compound	EAAC1	C6 glioma	Cortical tissues	
			Synaptosomes	Astrocytes[b]
D-aspartate	1.1 ± 0.5 μM	n.d.	2.8 μM[a]	78 μM
L-aspartate	2.0 ± 1.4 μM	n.d.	3.2 μM[a]	37 μM
DL-THA	3.7 ± 1.2 μM	6.1 ± 0.6 μM	2.3 ± 0.2 μM	35 μM
L-Cys	4.9 ± 2.2 μM	n.d.	1.8 μM[a]	27 μM
L-CCGIII	10 ± 3 μM[e]	13 ± 2 μM[d]	2.4 ± 1.5 μM	15 μM
L-*trans*-PDC	14 ± 3 μM[d]	14 ± 2 μM[d]	2.6 ± 0.5 μM	44 μM
L-CCGIV	171 ± 22 μM[c]	n.d.	48 ± 6 μM	660 μM
L-AP3	260 ± 30 μM	n.d.	100 μM[a]	n.d.
NMDA	290 ± 100 μM	n.d.	390 μM[a]	22% at 3 mM
L-CCGII	400 ± 40 μM	n.d.	2.2 μM (10%)[a] 490 μM (86%)	180 μM
Quisqualate	840 ± 390 μM	740 ± 220 μM	0.22 μM (8%)[a] 540 μM (86%)	59% at 3 mM
D-glutamate	1160 ± 330 μM[e]	1370 ± 240 μM[d]	280 ± 45 μM	n.d.
Dihydrokainate	1120 ± 200 μM[d]	1300 ± 240 μM[c]	110 ± 16 μM	6% at 3 mM
L-ODAP	52 ± 10% at 1 mM	n.d.	20% at 1 mM[a]	n.d.
α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid	50 ± 4% at 1 mM	n.d.	53% at 1 mM[a]	0% at 3 mM
L-AAD	39 ± 3% at 1 mM	49 ± 6% at 1 mM	870 ± 220 μM	1560 μM
Kainate	38 ± 7% at 1 mM	24 ± 12% at 1 mM	65 μM (71%)[a] 3180 μM (21%)	1670 μM
α-Methyl-DL-glutamate	21 ± 4% at 1 mM	n.d.	14% at 1 mM[a]	n.d.
L-CCGI	19 ± 4% at 1 mM	n.d.	16% at 1 mM[a]	0% at 3 mM
(±)-1-aminocyclopentane-trans-1,3-dicarboxylic acid	15 ± 9% at 1 mM	n.d.	38% at 1 mM[a]	10% at 3 mM
L-Homocysteate	11 ± 5% at 1 mM	19 ± 6% at 1 mM	46 μM (25%)[a] 1450 μM (76%)	36% at 3 mM

Values represent either K_i values or percent inhibition and are the mean ± standard error of at least three experiments.
[a] Data from Robinson *et al.* (6).
[b] Data for properties of transport in astrocyte-enriched cultures are from Garlin *et al.* (7).
[c] $p < 0.01$, [d] $p < 0.05$, [e] $p = 0.07$ compared with cortical synaptosomes (analysis of variance by Fisher's PLSD test).
n.d. = inhibitor not tested.

(6). Although most compounds (16 of 21) inhibited EAAC1-mediated L-[^{3}H]glutamate uptake with potencies similar to that previously reported for inhibition of cortical synaptosomal transport (within 3-fold), some compounds did show >3-fold selectivity. To rule out the possibility that experimental artifacts contributed to these pharmacological differences, inhibition of EAAC1-mediated and cortical synaptosomal transport was examined in parallel experiments. The concentration dependence for inhibition of EAAC1-mediated and cortical synaptosomal L-[^{3}H]glutamate transport by the two EAA analogues that were most different in these two systems are presented in Fig. 2 and compared with data obtained in C6 glioma. As previously reported, DHK inhibited L-[^{3}H]glutamate uptake into cortical synaptosomes, with a K_i value of 110 μM (Fig. 2A and Table 2). DHK was 10-fold less potent as an inhibitor of EAAC1-mediated transport, with a K_i value of 1120 μM. This value was comparable to that observed for inhibition of transport into C6 glioma (K_i = 1300 μM). Similarly, L*trans*-PDC was almost 5-fold more potent as an inhibitor of L-[^{3}H]glutamate transport into cortical synaptosomes (K_i = 2.6 μM) than into EAAC1-injected oocytes (K_i = 14 μM). L*trans*-PDC inhibited transport into C6 glioma with a potency similar to that observed for EAAC1-mediated L-[^{3}H]glutamate transport (K_i = 14 μM) (Fig. 2B and Table 2).

Western blot analyses. To verify that our EAAC1-injected oocytes express EAAC1-like immunoreactivity, we used the EAAC1 peptide-directed antibody (21) for Western blot analysis on proteins from EAAC1-injected and water-injected oocytes. In protein from oocytes injected with EAAC1 cRNA, the EAAC1 antibody recognized a protein at 77 kDa that was absent in protein from water-injected oocytes (Fig. 3A). This experiment was repeated three additional times, and the EAAC1 antibody recognized a protein with an average molecular mass of 80 ± 1 kDa in EAAC1-injected oocytes but did not recognize a protein from water-injected oocytes.

To determine which subtype or subtypes of glutamate transporters are expressed by C6 glioma, previously characterized antibodies directed against carboxyl-terminal peptides were used for Western blot analyses (Fig. 3B). As previously observed in brain homogenates (21), each of the antibodies recognized a broad band in crude synaptosomal membranes from cortex that was ~10–15 kDa larger than that predicted from the primary amino acid sequences (12–14). In cortical synaptosomal membranes, EAAC1-like immunoreactivity was 67 ± 1 kDa (seven experiments), GLT1-like immunoreactivity was ~72 kDa, and GLAST-like immunoreactivity was ~61 kDa. C6 glioma did not express GLT1- or GLAST-like immunoreactivity but did express EAAC1-like immunoreactivity. The EAAC1-like immunoreactivity in C6 glioma was 73 ± 1 kDa (seven experiments), which was ~6 kDa larger than EAAC1-like immunoreactivity observed in cortical synaptosomes (Fig. 3B).

To determine whether the differences in molecular mass of the various EAAC1-like immunoreactivities can be attributed to *N*-linked glycosylation, EAAC1-injected oocytes, C6 glioma, and cortical synaptosomes were treated with *N*-glycosidase F and subjected to Western blot analysis with the EAAC1 antibody (Fig. 3C, *lanes 9–11*). This treatment resulted in the appearance of bands that were 60 ± 2 kDa in

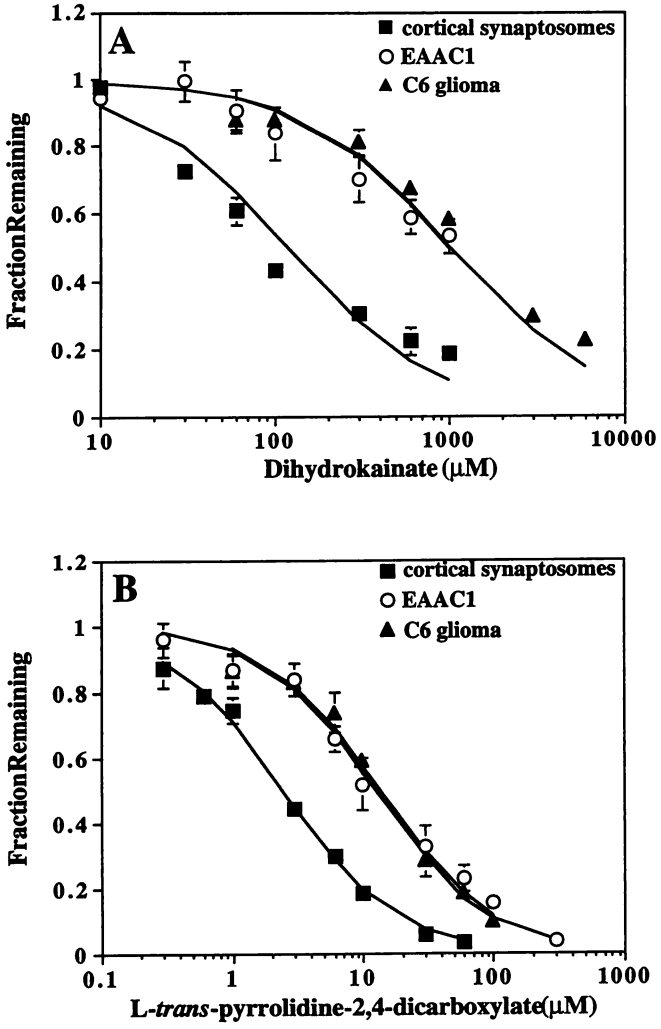

Fig. 2. Inhibition of L-[^{3}H]glutamate (0.5 μM) transport by DHK (A) and L*trans*-PDC (B). L-[^{3}H]glutamate transport was measured in the absence and presence of increasing concentrations of inhibitor. Data are the mean ± standard error of at least three independent experiments. *Solid lines*, theoretical curves with a Hill coefficient of 1 and K_i values of 120 μM (cortical synaptosomes), 1120 μM (EAAC1), 1300 μM (C6 glioma) for DHK and 2.5 μM (cortical synaptosomes), 13 μM (EAAC1), and 14 μM (C6 glioma) for L*trans*-PDC.

EAAC1-injected oocytes (five experiments), 57 ± 2 kDa in C6 glioma (four experiments), and 58 ± 2 kDa in cortical synaptosomes (four experiments).

Discussion

In the current study, the properties of glutamate transport mediated by the EAAC1 subtype expressed in *Xenopus* oocytes were characterized and compared with the properties of glutamate transport observed in synaptosomes, astrocyte-enriched cultures, and C6 glioma.

Although a previous report suggests that oocytes endogenously express glutamate transport (28), there was no detectable Na^+-dependent L-[^{3}H]glutamate transport in uninjected or water-injected oocytes as we previously reported (29). This observation indicates that oocytes are a suitable system for glutamate transport expression studies. Although these data suggest that EAAC1 is functioning as a homomeric protein in oocytes, it is not possible to rule out coassembly of EAAC1 with an endogenously expressed oocyte protein.

Based on the L-AAD sensitivity and DHK insensitivity of the rabbit homologue of EAAC1 (13), it might be concluded that the activity encoded by EAAC1 parallels the activity observed in rat cerebellar synaptosomes (5). Because of potential species differences, it may not be relevant to compare the transport properties of rabbit and human homologues with those properties observed in rat brain systems. Therefore, we chose to study the properties of glutamate transport in the rat homologue of EAAC1. In our initial characterization of EAAC1-mediated transport, kinetic differences were observed between transport into cerebellar synaptosomes and transport into EAAC1-injected oocytes (Table 1). The apparent K_m values for both L-[^{3}H]glutamate and Na^+ are 3–8-fold higher for transport into EAAC1-injected oocytes than for transport into cerebellar synaptosomes. This shift could be attributed to differences associated with measuring transport into the intact oocyte compared with synaptosomal membranes, but the potencies of inhibitors might be expected to shift as well. Compounds that have a broad range of selectivity (1–>60-fold) for inhibition of transport measured in cerebellar and cortical synaptosomes were tested as inhibitors of EAAC1-mediated transport. Many of the compounds that have <2-fold selectivity for inhibition of cerebellar or cortical synaptosomal transport, including DL-THA, D-aspartate, L-aspartate, and L-cysteine, have comparable K_i values for inhibition of rat EAAC1-mediated transport (Table 2, data for cerebellum not shown; see Refs. 5 and 6). In contrast to previously published data for the rabbit homologue, we found that compounds that selectively inhibit cerebellar transport are much less potent as inhibitors of EAAC1-mediated transport. For example, L-AAD competitively inhibited cerebellar transport with a K_i value of 31 μM (5) but inhibited EAAC1-mediated transport only 39% at 1 mM. L-Homocysteate competitively inhibited cerebellar transport with a K_i value of 42 μM (6) but inhibited EAAC1-mediated transport only 11% at 1 mM. L-CCGII competitively inhibited cerebellar transport with a K_i value of 4.2 μM (6) but inhibited EAAC1-mediated transport with a K_i value of 400 μM. α-Methyl-DL-glutamate inhibited cerebellar transport with a K_i value of 69 μM (6) but inhibited EAAC1-mediated transport only 21% at 1 mM. Therefore, these data provide strong evidence that the properties of the rat homologue of EAAC1 do not parallel the properties of cerebellar synaptosomal transport, even though EAAC1-like immunoreactivity is present in protein prepared from cerebellar synaptosomes.[1] Based on the L-AAD sensitivity of EAAT4-mediated transport activity and the cerebellar localization of EAAT4 mRNA (20), it appears that EAAT4 may account for the pharmacological properties observed in cerebellar synaptosomes (5, 6), but additional studies are needed.

In vivo, EAAC1-like immunoreactivity is localized exclusively to neurons (21). As might be predicted, transport expressed by EAAC1-injected oocytes did not resemble transport observed in astrocyte-enriched cultures (7). Most EAA analogues were ~3–70-fold more potent as inhibitors of EAAC1-mediated transport, with the exception of L-CCGII, which was more potent as an inhibitor of transport into

[1] L. A. Dowd, A. J. Coyle, J. D. Rothstein, D. B. Pritchett, and M. B. Robinson, unpublished observations.

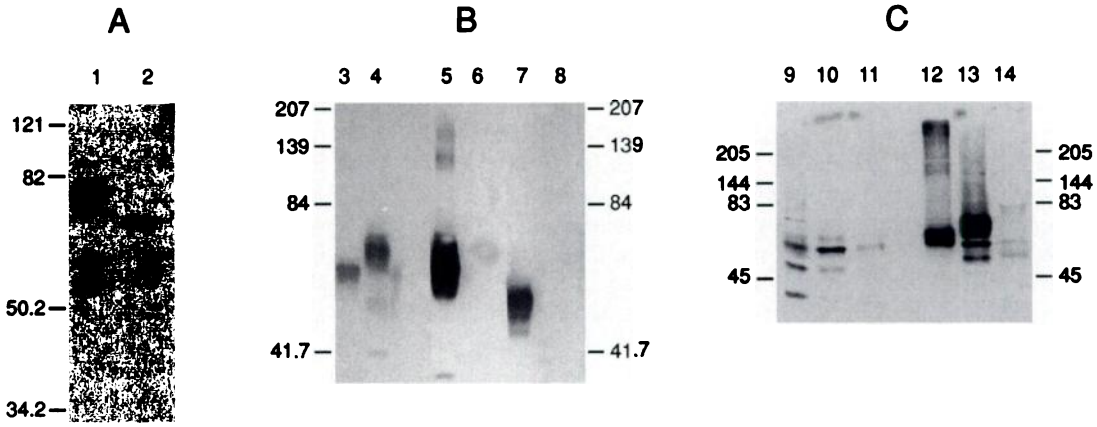

Fig. 3. A, EAAC1-injected oocytes and water-injected oocytes immunoblotted with the EAAC1 antibody. *Lane 1*, 50 μg of protein from EAAC1-injected oocytes. *Lane 2*, 50 μg of protein from water-injected oocytes. This figure shows a representative Western blot with EAAC1 immunoreactivity at 77 kDa. The average molecular mass for the immunoreactivity in EAAC1-injected oocytes is 80 ± 1 kDa (four experiments). B, Immunoreactivity for EAAC1, GLAST, and GLT1 subtypes of glutamate transporters in crude synaptosomal membranes and C6 glioma; 50 μg of cortical synaptosomal protein or 10 μg of C6 glioma protein were loaded per lane. *Lane 3*, cortical protein visualized with the EAAC1 antibody. *Lane 4*, C6 glioma protein visualized with the EAAC1 antibody. *Lane 5*, cortical protein visualized with the GLT1 antibody. *Lane 6*, C6 glioma protein visualized with the GLT1 antibody. *Lane 7*, cortical protein visualized with the GLAST antibody. *Lane 8*, C6 glioma protein visualized with the GLAST antibody. C, *Lane 9* (cortical protein), *lane 10* (C6 glioma protein), and *lane 11* (EAAC1-injected oocyte protein) depict immunoreactivity for EAAC1 after pretreatment with *N*-glycosidase F; 50 μg of EAAC1-injected oocyte protein, 50 μg of cortical synaptosomal protein, and 10 μg of C6 glioma protein were loaded per well. The EAAC1-like immunoreactivities in the three samples in this representative blot were 62 kDa. *Lane 12* (untreated cortical protein), *lane 13* (untreated C6 glioma), and *lane 14* (untreated EAAC1-injected oocyte protein) were examined in parallel. After Western blot analyses, the autoradiographic films were photographed, and the photographs were scanned as TIFF files onto a Macintosh computer. Migrations of molecular mass markers are provided as a reference.

astrocyte-enriched cultures (Table 2). For example, D-aspartate inhibited glial transport with a K_i value of 78 μM (7) and inhibited EAAC1-mediated transport with a K_i value of 1.1 μM. DHK did not inhibit glial transport (6% at 3 mM) (7) but did inhibit EAAC1-mediated transport with a K_i value of 1120 μM. L-CCGII inhibited glial transport with a K_i value of 180 μM (7) and inhibited EAAC1-mediated transport with a K_i value of 400 μM. This lack of pharmacological similarity suggests that EAAC1 does not contribute significantly to transport activity in astrocyte-enriched cultures. The pharmacological profile and glial localization of GLAST suggest that this subtype is responsible for the transport observed in astrocyte-enriched cultures (7, 15).

In vivo, the EAAC1 subtype is highly abundant in forebrain regions, with the greatest level of expression in hippocampus (21). Based on these data, it seems that EAAC1 might be responsible for the transport previously characterized in cortical synaptosomes (5, 6). Although the properties of EAAC1-mediated transport are similar to those observed in cortical synaptosomes, there are differences. The kinetic constants for both L-[^{3}H]glutamate and Na^+ were 3-fold higher in EAAC1-injected oocytes than in cortical synaptosomes (Table 1). Sixteen of the 21 EAA analogues inhibited transport in EAAC1-injected oocytes with K_i values within 3-fold of those observed in cortical synaptosomes (Table 2). The potencies of many of the compounds for inhibition of cortical synaptosomal transport were re-examined in the current study, and all of the values were within 15% of that previously reported. However, five of the EAA analogues were ≥3-fold more potent at inhibiting transport into cortical synaptosomes than at inhibiting transport into EAAC1-injected oocytes. For example, DHK inhibited cortical transport with a K_i value of 110 μM and inhibited EAAC1-mediated transport with a K_i value of 1120 μM. These data suggest that EAAC1 does not account for the pharmacological properties of transport observed in cortical synaptosomes when these properties are determined at low concentrations of L-[^{3}H]glutamate (0.5 μM). It is possible that the pharmacology of these transporters may by affected by the differences in EAAC1 glycosylation.

Although the Western blot analyses indicated that EAAC1-, GLT1-, and GLAST-like immunoreactivities are present in the cortical synaptosomal membranes (Fig. 3), the densities of these immunoreactivities did not allow us to define the relative contributions of these three transporters to transport activity in this preparation. The presence of the glial transporters GLT1 and GLAST may be accounted for by glial contamination of these preparations (30). Although the three subtypes are present in cortical synaptosomes, some of these transporters may be localized to unsealed membrane compartments. The pharmacological comparisons indicate that these transporters do not account for the properties observed at low concentrations (0.5 μM) of L-[^{3}H]glutamate (see above). It is possible that 0.5 μM glutamate is not cleared by the different transporters to the same extent because of differences in K_m values. Therefore, based on pharmacology alone, these transporters may not contribute to the activity previously observed in cortical synaptosomes at low L-[^{3}H]glutamate concentrations (5, 6) but may contribute to the activity at higher L-[^{3}H]glutamate concentrations. However, a comparison of the capacities for transport in C6 glioma and synaptosomes relative to a comparison of the amounts of immunoreactivity provides evidence that EAAC1 may not contribute significantly to transport in synaptosomes. C6 glioma express ≥5-fold more immunoreactivity than that observed in synaptosomes (Fig. 3), but the capacity for transport is less than one fifth of that observed in synaptosomes (C6 glioma, 430 ± 80 pmol/mg/min; synaptosomes, 2700 pmol/mg/min; Refs. 5 and 6). If one assumes that the relationship of immunoreactivity to transport activity is the

same in C6 glioma and cortical synaptosomes, then <5% of the transport activity in cortical synaptosomes can be attributed to EAAC1. However, the level of surface expression and/or other factors may affect the ratio of immunoreactivity to transport activity in these two different systems. For example, it is possible that in C6 glioma, EAAC1-like immunoreactivity is present on intracellular compartments and that the actual number of functional surface transporters is much lower, accounting for the lower capacity in these cells. The relative contributions of these transporters to the clearance *in vivo* cannot be determined from these studies and may vary depending on the extracellular concentrations of EAAs.

C6 glioma were originally thought to represent a mature astrocyte type of cell line (31), although more recent evidence suggests that C6 glioma most closely represent the less-differentiated stem cells that develop into either astrocytes or oligodendrocytes (32). The expression of these cell type properties can be modified by a variety of culture conditions, including high cell density, which leads to glial differentiation (33–35). Previous studies demonstrated that C6 glioma endogenously express SDHA glutamate transport activity (36, 37). In the current study, the properties of EAAC1-injected oocytes parallel the properties of glutamate transport observed in C6 glioma. In EAAC1-injected oocytes and C6 glioma, the kinetic constants for both L-[^{3}H]glutamate and Na^+ were within 2-fold of each other (Table 1). Unlike the differences between EAAC1-mediated transport and cortical synaptosomal transport, DHK, L*trans*-PDC, L-CCGIII, and D-glutamate inhibited transport into C6 glioma and transport into EAAC1-injected oocytes with K_i values that were within 40% of each other (Table 2). Although previous studies suggest that C6 glioma express GLT1 (38), we found that our C6 glioma exclusively expressed EAAC1-like immunoreactivity and not GLT1- or GLAST-like immunoreactivity (Fig. 3). Our data agree with those of Palos *et al.* (39), who reported that C6 glioma express EAAC1 mRNA but do not express GLAST or GLT1 mRNA. It is possible that sublines of C6 glioma express different subtypes of glutamate transporters.

The molecular mass of EAAC1 in C6 glioma is ~6 kDa larger than the protein recognized in crude cortical synaptosomes, whereas the molecular mass of EAAC1 in EAAC1-injected oocytes is ~13 kDa larger than the protein recognized in crude cortical synaptosomes (Fig. 3C). The rat homologue of EAAC1 has three consensus sites for *N*-linked glycosylation, all of which are predicted to be extracellular based on hydrophobicity plots. *N*-Glycosidase F treatment of EAAC1-injected oocytes, C6 glioma, and cortical synaptosomes provided evidence that the difference in molecular mass is due to differences in glycosylation (Fig. 3C), which has also been observed with dopamine and serotonin transporters (22, 40). It is unclear whether this difference in glycosylation contributes to the pharmacological differences observed in C6 glioma and synaptosomal membranes.

Kanai and Hediger (13) originally characterized the rabbit homologue of EAAC1 by measuring L-glutamate-evoked currents in oocytes injected with cRNA. Arriza *et al.* (18) expressed the human homologue in COS-7 cells and in oocytes. Although several properties of the rat homologue of EAAC1 are comparable to those previously reported for the rabbit and human homologues, there are some notable differences. The three species homologues convey Na^+-dependent L-glutamate transport that is saturable and consistent with a single site (K_m = 12–62 μM). Although DHK does not inhibit transport mediated by the rabbit (<10% inhibition at 1 mM) (13) or human homologues (K_i > 3 mM) (18, 19), DHK inhibited transport mediated by the rat homologue with a K_i value of 1120 μM (Table 2). L-AAD inhibited transport-mediated by the rabbit homologue with an IC_{50} value of 165 μM (13), inhibited transport mediated by the human homologue with a K_i value of ~1900 μM (19), and inhibited transport mediated by the rat homologue with a K_i value of >1 mM (39% inhibition at 1 mM; Table 2). Although experimental variables such as choice of expression system may be responsible for these pharmacological discrepancies, these discrepancies may be due to species differences or to the presence of endogenous glutamate transporters. For example, COS-7 cells express GLAST-like immunoreactivity.[2] A recent study with serotonin transporters provides a precedent for the species differences. Barker *et al.* (22) demonstrated that the tricyclic antidepressant imipramine is 6-fold more potent as an inhibitor of human serotonin transport than of rat serotonin transport and that the stimulant D-amphetamine is 3.5-fold more potent as an inhibitor of rat serotonin transport than of human serotonin transport. If there are species differences in the pharmacology of these highly homologous glutamate transporters, then this may provide insights into the amino acid residues that are responsible for inhibitor recognition.

In conclusion, with the use of Western blot analyses, we demonstrated that cortical synaptosomes express EAAC1-, GLT1-, and GLAST-like immunoreactivities but that the relative abundance of each subtype is not known. C6 glioma, however, endogenously expressed EAAC1-like immunoreactivity and did not express GLT1- or GLAST-like immunoreactivity. Our results suggest that EAAC1 does not account for the pharmacology previously observed in cortical synaptosomes. Based on the pharmacology and tissue distribution of EAAC1, GLT1, and GLAST, it seems that additional glutamate transporter subtypes exist. A subtype with forebrain localization and sensitivity to DHK (K_i value of 110 μM) and L-AAD (K_i value of 870 μM) has not been isolated.

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