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Characterization of Na^+-Coupled Glutamate/Aspartate Transport by a Rat Brain Astrocyte Line Expressing GLAST and EAAC1

G.A. Kimmich, J. Roussie, M. Manglapus,* J. Randles
Department of Biochemistry and Biophysics, and *Department of Ophthalmology School of Medicine and Dentistry, University of Rochester, Rochester, NY 14642, USA

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Abstract. D-Aspartate (D-Asp) uptake by suspensions of cerebral rat brain astrocytes (RBA) maintained in long-term culture was studied as a means of characterizing function and regulation of Glutamate/Aspartate (Glu/Asp) transporter isoforms in the cells. D-Asp influx is Na^+-dependent with K_m = 5 μM and V_{max} = 0.7 nmoles · min^{-1} · mg $protein^{-1}$. Influx is sigmoidal as f[Na^+] with $_{Na^+}K_m$ ~ 12 μM and Hill coefficient of 1.9. The cells establish steady-state D-Asp gradients >3,000-fold. Phorbol ester (PMA) enhances uptake, and gradients near 6,000-fold are achieved due to a 2-fold increase in V_{max}, with no change in K_m. At initial [D-Asp] = 10 μM, RBA take up more than 90% of total D-Asp, and extracellular levels are reduced to levels below 1 μM. Ionophores that dissipate the $\Delta\mu_{Na^+}$ inhibit gradient formation. Genistein (GEN, 100 μM), a PTK inhibitor, causes a 40% decrease in D-Asp. Inactive analogs of PMA (4α-PMA) and GEN (daidzein) have no detectable effect, although the stimulatory PMA response still occurs when GEN is present. Further specificity of action is indicated by the fact that PMA has no effect on Na^+-coupled ALA uptake, but GEN is stimulatory. D-Asp uptake is strongly inhibited by serine-*O*-sulfate (S-O-S), threohydroxy-aspartate (THA), L-Asp, and L-Glu, but not by D-Glu, kainic acid (KA), or dihydrokainate (DHK), an inhibition pattern characteristic of GLAST and EAAC1 transporter isoforms. mRNA for both isoforms was detected by RT-PCR, and Western blotting with appropriate antibodies shows that both proteins are expressed in these cells.

Key words: Astrocytes — Neurotransmitter transport — Glutamate/aspartate transport — GLAST — EAAC1 — Na^+-coupled glutamate transport — PKC activation of Glu/Asp transport — Genistein

Correspondence to: G.A. Kimmich

Introduction

One of the notable accomplishments in neuroscience of the past decade is recognition of the vital role neurotransmitter transport systems play in sustaining function of the central nervous system (for reviews *see* Amara & Kuhar, 1993; Lester et al., 1994). Extracellular transmitter concentrations in the synapse are the result of cellular dynamics that involve not only rates of production and release by presynaptic neurons, but also the rate at which transmitter molecules are taken up by other cells adjacent to the synapse. Each event and participating cell type plays a role in modifying peak and steady-state transmitter concentration and the rates at which such levels are achieved. Hence, each event is a site of potential control for CNS function associated with transmitter action. Drugs that inhibit transport systems for specific transmitters have gained prominence in the clinical setting because of their efficacy in correcting malfunction. Notable among these are the anti-depressant actions of selective serotonin reuptake inhibitors (SSRI). New clinical applications are possible as additional insight is achieved regarding modulation of neurotransmitter 'recapture' events.

The role of glial cells in glutamate uptake was a matter of early debate because neuronal cells cannot catalyze net glutamate synthesis when glucose is the sole available substrate (Shank et al., 1985). If astrocytes capture glutamate, the presynaptic neuron faces a carbon deficit for sustaining glutamate resynthesis. This implies a return of carbon from the glia in a form that does not induce or interfere with the activity of glutamate receptors on pre- or post-synaptic neuronal cells (Shank et al.,

1985; Waniewski & Martin, 1986; Hertz & Schousboe, 1986; Kaufman & Driscoll, 1992). That glial cells indeed capture glutamate has been shown by selective knockdown of astrocytic glutamate transporters (GLT1 or GLAST) with antisense mRNA, which induces overt malfunction of locomotor systems in rats (Rothstein et al., 1996). Knockdown of the neuronal transporter (EAAC1) causes less pronounced ill effects than those associated with loss of either astrocytic isoform. The results leave little doubt as to the important role that astrocytic transporters play in the dynamics that 'set' resting synaptic glutamate levels. Glial and neuronal transport systems are both of fundamental importance in allowing the prominent role for glutamate in excitatory CNS neurotransmission events (Rothstein et al., 1994; Kanai et al., 1995; Kondo et al., 1995; Lehre et al., 1995; Velaz-Faircloth et al., 1996).

Although glutamate capture by glia requires a more complex flow of molecular traffic, it serves a valuable purpose because glutamate is a potent neurotoxin if it remains in the synapse at elevated levels for even short intervals (Choi, 1988; Rothstein et al., 1993; Schousboe & Frandsen, 1995). Glial cell participation in the reuptake process helps ensure rapid restoration of normal steady-state glutamate levels in the synapse, hence diminishing neurotoxic risk.

Model systems for characterizing glial glutamate transport events, and for evaluating cellular signals important in modulating their activity are important because of the prominent role such systems play in maintaining low resting glutamate levels. In this report we characterize properties and regulatory aspects of excitatory amino acid transport in a rat brain astrocyte (RBA) cell line that expresses the GLAST (Storck et al., 1992, Klockner et al. 1993) and EAAC1 GLU/ASP transporter isoforms. To our knowledge this is the first example of a long-term astrocyte culture that manifests GLAST transporter activity. The results also show that the RBA line retains transporter function modulated by PKC and PTK effectors. PKC activation induces an increased transport V_{max} with no change in K_m. This model system may prove to be useful for discerning whether protein phosphorylation plays a direct role in activation of transporter already in the membrane, or serves as a signal for transporter trafficking to the plasma membrane, and whether both isoforms respond similarly.

Methods and Materials

Rat Brain Astrocyte Culture and Harvest

The cells used for all experiments were from a rat brain astrocyte line (RBA-1) that was the generous gift of Dr. Shey-Shing Sheu, Department of Pharmacology and Physiology, University of Rochester Medical Center. The line originated from a primary culture of astrocytes derived from rat cerebrum and was developed through successive cell passages without oncogen or other chemical treatment. The line has been maintained in culture for more than 200 passages, including a four-year interval in our own laboratory. The cells retain stellate morphology and stain positively with antibody to glial fibrillary acidic protein (anti-GFAP) (Peng and Sheu, 1994; Kimmich et al., *unpublished results*). The cells were grown in a Dulbecco's modified Eagle's media (DMEM) in 1:1 ratio with Ham's F-10 nutrient mixture (both from GIBCO) supplemented with 10% fetal bovine serum (GIBCO) and with penicillin (100 units/ml) and streptomycin (100 μM) to prevent bacterial contamination. Cultures were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air. The plates were provided with fresh medium every second or third day. Cells were 'passed' to fresh plates after 4–6 days in culture, and reach confluency on 100 mm culture plates after 5 days when seeded at 5% of confluent density.

Cells are passed to new plates in suspension by replacing the culture medium with 5 ml of fresh medium and gently refluxing the medium in and out of a 5 ml pipette while directing the fluid stream against the bottom surface of the culture plate. No proteolytic enzymes or chelators are necessary to release cells from the plates. Cell suspensions were centrifuged at 350 × g for three minutes to create a loosely packed cell pellet, supernatants were aspirated off, cells were resuspended in fresh medium, and new plates were initiated at 5% of the confluent cell density.

For transport studies, cells were harvested by a procedure similar to that described for passaging. Cell harvest was in a medium containing (in mM): 140 NaCl, 2 K_2HPO_4, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-TMA buffer (pH 7.4), 5 glucose, and 1 mg/ml BSA. Plates chosen for harvest were slightly preconfluent (80–90%) or had reached confluency in the previous 12 hours. After centrifugation (350 × g), the cells were resuspended in the same medium at approximately 20 mg cell protein/ml. Protein was determined by the biuret procedure in comparison to bovine serum albumin standards. Confluent cultures yield ~2 mg cell protein per 100 mm plate. Cell suspensions were kept on ice until used. All experiments were initiated within 30 min, and completed within 2 hr from the time of cell harvest.

After every 15–20 passages, cultures were discarded and new cultures were started from frozen cell stocks that had been carried through fewer cell passages from the time of primary culture. All experiments reported here were accomplished with cells between passages 208–225. Frozen cell stocks were established by swirling tubes of cells suspended in growth medium with 10% dimethylsulfoxide (DMSO) in liquid nitrogen to ensure rapid freezing. Frozen stocks were stored in liquid nitrogen.

Aspartate Transport

The cells were incubated at 37°C for 20 min prior to initiating each experiment to allow thermal equilibration, and to allow the cells to establish stable electrochemical ion gradients. Transport properties were assessed using procedures established in our laboratory for similar work with suspensions of freshly isolated intestinal epithelial cells (Kimmich & Randles, 1984; Restrepo & Kimmich, 1985a,b; Wingrove & Kimmich, 1987, 1988; Kimmich, 1990) and cultured LLC-PK1 renal epithelial cells (Kimmich, Randles & Wilson et al., 1994; Wilson & Kimmich, 1996). All long-term experiments (40–120 min) were initiated by adding 0.1 ml of warmed cell suspension to 2.2 ml of 'transport' medium at 37°C. Unless otherwise noted, the transport medium consisted of the harvesting medium with 10 μM D-aspartate (D-Asp) and 0.2 μC of ^{3}H-D-Asp. In protocols requiring zero or low [Na^+], tetramethylammonium chloride (TMA-Cl) was used to replace NaCl. Influx of 10 μM D-Asp in Na^+-free medium is <2% of that observed at 140 mM Na^+, and is linear with D-Asp concentration. The difference ± Na^+ was taken as a measure of Na^+-dependent D-Asp uptake.

For unidirectional influx measurements (30–60 sec) 0.1 ml of cell suspension was added to 0.9 ml of transport medium, and ^{3}H-D-Asp was increased to 2.0 μC/ml. Experimental beakers were clamped in a stainless steel tray oscillating at 110 cycles per min in a 37°C water bath to ensure that the cells remained uniformly suspended during the incubation and sampling interval. D-Asp was used as the test substrate because it is a substrate for all glutamate transport systems that have been characterized (CNS and non-CNS), but it is not metabolized by astrocytes (Erecinska et al., 1986).

Aliquots (100 μl) were taken from the incubation beakers at timed intervals. The aliquots were diluted immediately into 4 ml of ice cold incubation medium without D-Asp. The diluted samples were centrifuged for 25 sec at 1,000 x g in an Adams MHCT II centrifuge. Supernatants were aspirated off and discarded, or in some cases saved for measurement of ^{3}H-D-Asp remaining in the extracellular incubation medium. The cell pellets were resuspended in 2 ml of chilled wash medium. The resuspended cells were again centrifuged (25 sec at 1,000 × g), the supernatant was removed, and the cell pellets were dissolved in 4 ml of Ecoscint (National Diagnostics) prior to measuring isotope by liquid scintillation. In some cases, supernatant samples after the initial centrifugation were counted to determine extracellular ^{3}H-D-Asp remaining after various uptake intervals.

Replicate samples for trials performed under identical conditions, give uptake values for D-Asp that typically differ by <5% for cells taken from the same suspension. Data taken from different cell preparations show a wider variation (20–30%) for otherwise similar trials. This relates, in part, to differences in transport V_{max} which show similar percent changes depending on degree of cell confluency at time of harvest. All experiments were repeated at least three times with results similar to those shown. Due to the variation in transport kinetics among cell populations, representative results derived from single suspensions are shown in some cases, as stated in the Figure legend. Transport agonists or antagonists induce the same percentage change in D-Asp uptake when comparing different cell preparations, even if 'Control' uptake rates differ.

Intracellular Space

Intracellular space for each cell preparation was measured by allowing astrocytes incubated at 37°C to equilibrate with 100 μM ^{14}C-3-*O*-methylglucose (3-OMG) in the usual transport medium. This methylsugar cannot be metabolized (Csaky & Thale, 1960), yet is allowed entry to the cell via the 'facilitative' sugar transport systems that allow passive entry of glucose. Astrocytes do not have Na^+-dependent sugar transport capability, and do not accumulate 3-OMG against a concentration gradient. After equilibration, the intracellular volume was determined from CPM taken up/CPM per μl of ^{14}C-3-OMG in the incubation medium (Kimmich, 1975; Randles & Kimmich, 1978). A mean intracellular volume 2.65 ± 0.05 μl per mg of cell protein ($n = 9$) was obtained. This value is independent of 3-OMG concentration over a 100-fold range (0.1–10 mM).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The glutamate transporter mRNA produced by the RBA cell line was determined by RT-PCR. Total RBA RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH). Synthesis of cDNA from the mRNA fraction was performed using 1 μg total RNA, 1 μg of oligo-(dT) (15mer, Promega, Madison, WI) as primer, and 200 units of Superscript II RNase H^- Reverse Transcriptase (Gibco BRL, Grand Island, NY) for 1 hr at 42°C. Parallel reactions without reverse transcriptase were used to ensure that amplification products were derived from RNA and not due to contamination with genomic DNA. A 1 μl aliquot of cDNA products synthesized was subsequently used as template for 30 cycles of PCR with a panel of primers for specific transporter isoforms (*see* below). Primers were designed to amplify fragments selectively from the respective cDNAs of GLAST, GLT1, EAAC1, and EAAT4. PCR was performed in a 25 μl volume containing 0.2 μM upstream and downstream primers and 0.5 units of Platinum *Taq* DNA Polymerase (Gibco BRL, Grand Island, NY). PCR products in a 5 μl aliquot were then separated by ethidium-bromide agarose gel electrophoresis.

PCR Primers

Primer specifications and amplification fragments chosen for each transporter cDNA were as follows: for GLAST, a 586 bp product spanning nucleotides 382 to 967 (GeneBank Accession No. X63744) was amplified using 5′GATTTGCCCTCCGACCGTAT3′ as upstream primer and 5′ATGTTTCCGATCACGAAGCC3′ as downstream primer; for GLT1, a 587 bp product spanning nucleotides 239 to 825 (GeneBank Accession No. X67857) was amplified using upstream primer 5′CTCACTGACTGTGTTTGGTG3′ and downstream primer 5′CTAAGACATTCATCCCGTCC3′; for EAAC1, a 432 bp product spanning nucleotides 375 to 806 (GeneBank Accession No. U39555) was amplified using upstream primer 5′GTCATTCTGCCACTGATTAT3′ and downstream primer 5′GATGCCGTCTGAGTACAG3′; and for EAAT4, a 495 bp fragment spanning nucleotides 757 to 1251 (GeneBank Accession No. U89608) was amplified using upstream primer 5′CGAGTGGTAACAAGGACGAT3′ and downstream primer 5′GTTCCGATGTGTGACAAGG3′.

Unique restriction endonuclease cut sites were identified within each amplification product to allow production of digestion products of predictable size that were used as further proof that specific amplification had been achieved. Amplified fragments were digested with the appropriate endonuclease and the digestion products analyzed by ethidium bromide agarose gel electrophoresis.

Western Blotting

Confluent cell cultures were washed three times in ice-cold PBS (in mM: 138 NaCl, 2.7 KCl, 7.6 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.4) and lysed in 1 ml of ice-cold RIPA buffer (in mM: NaCl, 50 Tris-HCl pH 7.4, 1 EDTA, 1 PMSF, 1 NaF, 1 Na_3VO_4, 1% NP-40, 0.25% Na-deoxycholate, and 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Lysates were incubated with end-over-end mixing at 4°C for 20 min to extract cellular proteins and particulate matter was cleared by centrifugation at 14,000 × g for 20 min at 4°C. Protein was measured by the Lowry assay (Lowry et al, 1951). Protein extracts from whole rat brains were prepared in similar manner following tissue homogenization.

Cell proteins were resolved by SDS-PAGE on 8% polyacrylamide gels. Protein extracts were diluted 1:5 in loading buffer (10% glycerol, 62.5 mM Tris-HCl pH 8.8, 100 mM DTT, 2% SDS, after dilution) and incubated at 37°C for 30 min. Proteins were electroblotted from the gels onto PVDF membranes (Immobilon P, Millipore, Bedford, MA). Blots were blocked for 20 min in 3% nonfat dry milk/PBS and then incubated for 1 hr with primary antibodies diluted in 3% milk/PBS. Extensively characterized primary antibodies directed toward the C-terminal tail of EAAC1 and GLT1 and the N-terminal tail of GLAST were the generous gift of Dr. Jeffrey D. Rothstein (Johns Hopkins University, Baltimore, MD) (Rothstein et al, 1994; Rothstein

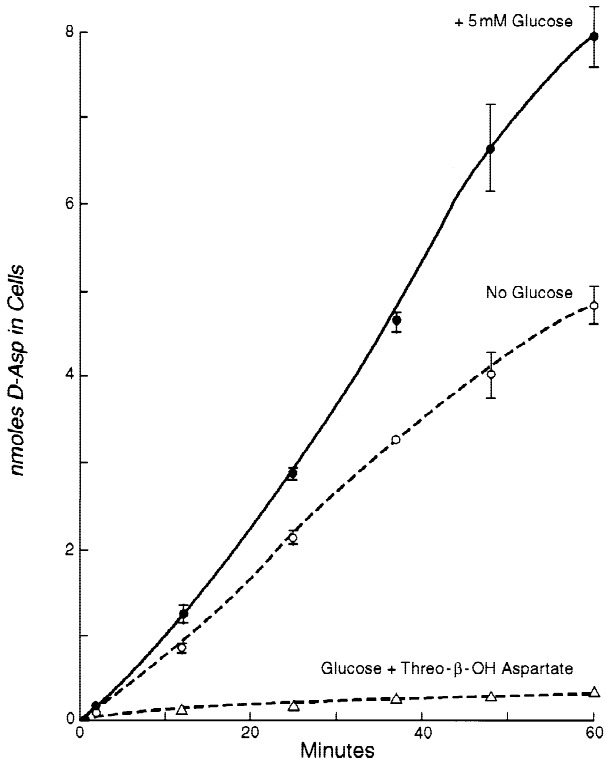

Fig. 1. Effect of 5 mM glucose and 200 μM threo-β-hydroxy-aspartate (THA) on the uptake of 10 μM D-Asp by a suspension of rat brain astrocytes. The sodium concentration was 140 mM in all cases. Each experimental run had 0.6 mg of cell protein per ml of incubation medium (0.06 mg protein per 100 μl sample).

et al, 1996). The anti-EAAC1 and anti-GLAST antibodies were used at a dilution of 1:200, and the anti-GLT1 antibody at 1:10,000.

Following incubation with primary antibody, the blots were washed twice in 0.1% Tween 20/PBS for 10 min, and then incubated 1 hr with HRP-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:5000 in 0.1% Tween 20/3% milk/PBS. Blots were washed twice in 0.1% Tween 20/PBS, then incubated 5 min with ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ) prior to exposure to photographic film.

Results

Figure 1 shows the results for D-Asp uptake by RBA cells during a one hour incubation. Absence of glucose compromises D-Asp uptake, although the cells still retain approximately 50% of the uptake capability observed with glucose present. When 200 μM THA is included in the medium, more than 98% of the total D-Asp uptake is inhibited. THA is a competitive inhibitor (K_i = 20–30 μM) of Na^+-coupled dicarboxylic amino acid transport systems (Flott & Seifert, 1991; Storck et al., 1992; Arizza et al., 1994).

Short term D-Asp uptake is linear with time for at least an 8 min incubation interval as shown in Fig. 2. Slope of the short-term uptake vs time relationship provides a measure of the unidirectional influx of D-Asp into the cells. Influx is directly proportional to the amount of cell protein over the range from 0.5 to 2.0 mg/ml (*data not shown*). Influx as a function of [D-Asp] is hyperbolic with a least-squares best fit to the Hill equation when $n = 1.0$. An Eadie-Hofstee plot of the influx data indicates a $_{Asp}K_m$ value of 4.8 μM, and transport V_{max} of 0.68 nmoles · min^{-1} · mg $protein^{-1}$ (Fig. 2*B*). Data obtained for three different cell preparations yielded K_m values near 5 μM (range 4–6 μM), indicative of high affinity D-Asp transport. Values for V_{max} ranged from 0.5 to 0.9 nmole · min^{-1} · mg $protein^{-1}$ for the three cell preparations.

Unidirectional influx of D-Asp to the cells is highly dependent on Na^+ concentration in the incubation medium as shown in Fig. 3*A*. At low [Na^+] uptake is sigmoidal, as shown in Fig. 3*B* and in the inset. Least squares fitting of influx data to the Hill equation for three separate experiments yields $_{Na}K_m$ values of 10–15 mM, and Hill coefficient of 1.9.

Intracellular volume, in conjunction with the D-Asp uptake data, was used to calculate the magnitude of steady-state aspartate gradients established by the astrocytes. For this purpose it is necessary to measure the pronounced change in extracellular D-Asp concentration that occurs during the uptake interval in addition to intracellular concentration. Figure 4 shows that astrocytes incubated with 10 μM D-Asp require approximately 60 min before achieving steady-state uptake. Typically, the cells accumulate 70–90% of the total aspartate available in the medium during this time, depending on cell density, even though intracellular astrocyte volume is a small percentage (0.25%) of the extracellular volume. In the four experiments summarized in Fig. 4, the cells established a mean D-Asp gradient of 1,500-fold after a one hr incubation interval, and the gradient was sustained for another 60 min. Data from a similar experiment performed at an initial D-Asp of 5 μM is provided in Table 1. It also shows the decrease in extracellular [^{3}H-D-Asp] as a function of incubation time, in comparison to values that were calculated by subtracting the observed ^{3}H-D-Asp uptake from the initial extracellular concentration. As shown, there is excellent agreement between the measured and calculated values. Both approaches show that the astrocytes establish submicromolar steady-state extracellular [D-Asp], and that gradients of ~3,000-fold are established under these conditions.

Capability of the RBA cells for establishing D-Asp gradients is partially sensitive to oligomycin, an inhibitor of mitochondrial oxidative phosphorylation. Better inhibition can be achieved with the use of ionophores such as gramicidin or nigericin that dissipate transmembrane gradients for monovalent cations. The ionophores and oligomycin are particularly potent when used together, as shown in Table 2. The potent inhibition under these conditions is consistent with transport capability that requires maintenance of a $\Delta\mu_{Na^+}$ across the plasma membrane for concentrative D-Asp uptake. The rate of ATP

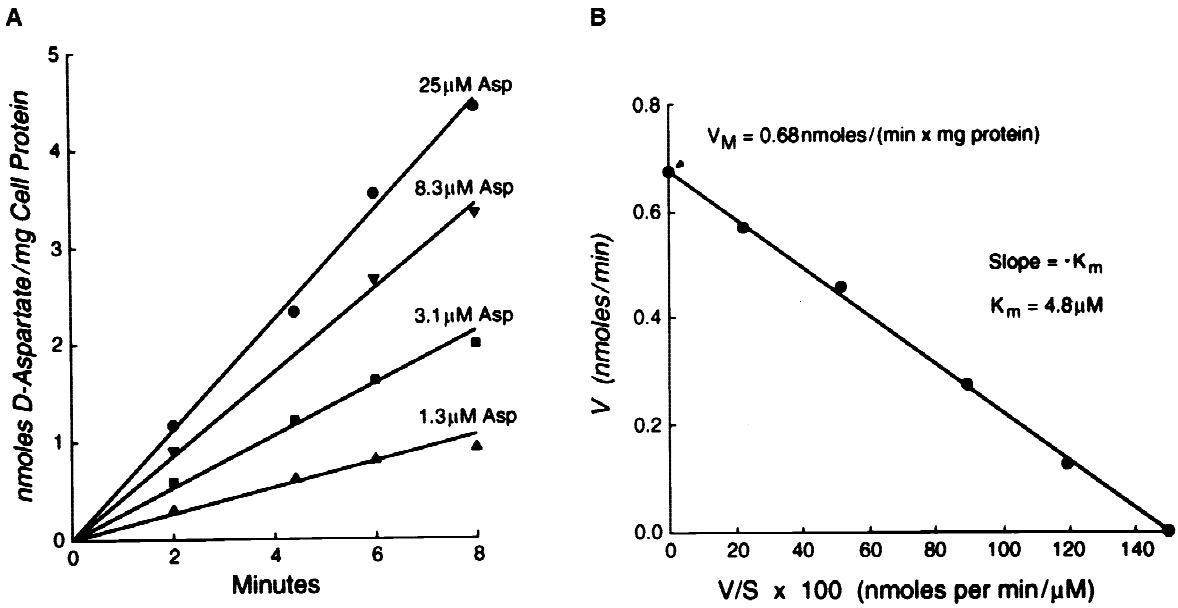

Fig. 2. (*A*) Unidirectional influx of D-Asp into rat brain astrocyte suspensions for several different concentrations of D-Asp. The Na^+ concentration was 140 mM in each case, and the cell protein concentration was 0.6 mg protein per ml. (*B*) Eadie-Hofstee plot of the data (*A*), showing a K_m for aspartate of 4.8 μM and a V_{max} of approximately 0.7 nmoles · min^{-1} · mg $protein^{-1}$.

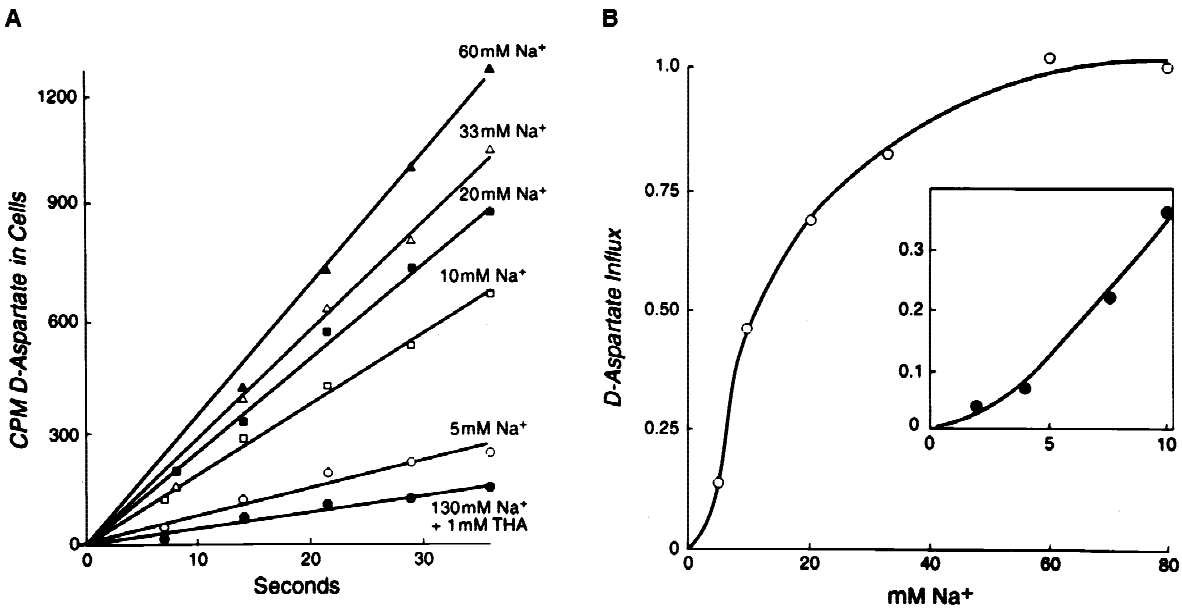

Fig. 3. (*A*) Unidirectional influx of D-Asp into rat brain astrocytes at several different Na^+ concentrations. The astrocytes were harvested at 140 mM Na^+, the suspensions were centrifuged and the cell pellets resuspended in a medium in which TMA^+ replaced all but 5 mM of the Na^+. The cells were incubated at 37°C for 20 min and then a 100 μl aliquot of the suspension was introduced to 0.9 ml of medium in which the Na^+ concentration was chosen to yield the final concentration shown for each experimental run (TMA^+ was used to maintain isosmotic conditions in each case). (*B*) D-Asp influx as a function of extracellular [Na^+]. The inset shows data from a separate experiment for 2–10 mM Na^+. The solid line in (*A*) shows a computer-generated fit of the data to the Hill equation with a K_m for Na^+ of 11 mM and a Hill coefficient of 1.9. For the inset the fit shown is for a $_{Na^+}K_m$ of 14 mM and a Hill coefficient of 1.9.

production from glycolysis cannot sustain Na^+-pump activity to maintain even minimal transport capability, when $\Delta\mu_{Na^+}$ is continually dissipated by ionophores.

Sensitivity of D-Asp uptake to various inhibitors of Na^+-coupled glutamate transport systems or to substrates known to compete for dicarboxylic amino acid transport systems was also evaluated. The K_i value calculated for each test agent from its' effect on the initial rate of up-

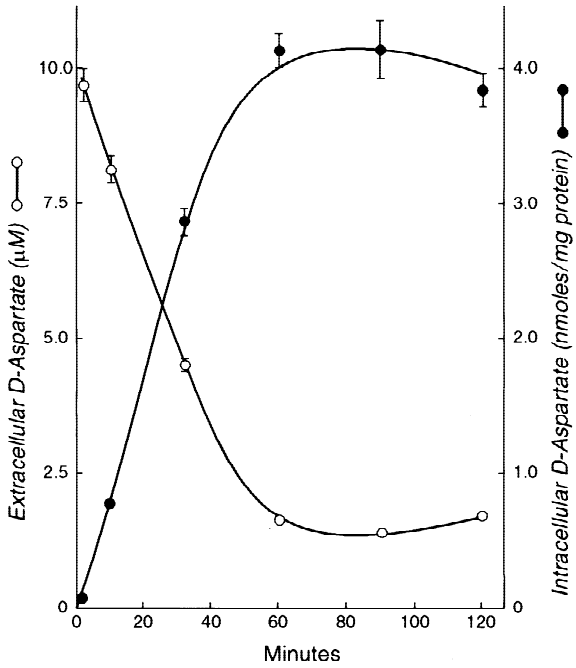

Fig. 4. Uptake of 10 μM D-Asp by rat brain astrocytes (1.0 mg cell protein/ml), and decrease in extracellular [D-Asp] as a function of time. Mean values ± SEM for three separate experiments are shown. Error bars not shown are smaller than the symbol used for the mean value.

take of 10 μM ^{3}H-D-Asp is shown in Table 3. The L- or D-isomer of aspartate (50 μM) each inhibit about 75% of 10 μM D-Asp influx, while 500 μM of either isomer causes >95% inhibition. The K_i values (5.7 and 8.2 μM) calculated for both test concentrations of D-Asp show good agreement with the K_m for D-Asp (~5 μM) determined by direct measurement (Fig. 2*B*). L-Asp showed a similar K_i (6 μM) to that of D-Asp, indicating that the transporter has nearly identical affinity for the two Asp isomers. On the other hand, L- and D-Glutamate are markedly different in inhibitory potency. The K_i for L-Glut (20 μM) is ~3-fold higher than for L- or D-Asp, but that for D-Glut is over 100 times higher (>700 μM), indicating very limited interaction for this substrate with the transporter. It is noteworthy that kainic acid (KA) and dihydrokainate have no detectable inhibitory effect at concentrations 25-fold higher than the reported K_i for these agents on the astrocytic GLT1 glutamate transporter (20 μM). At the other extreme, threo-hydroxyaspartate and serine-O-sulfate, two known competitive inhibitors of the glutamate transporter family, have K_i values similar to those for aspartate and glutamate, respectively.

RBA cells incubated with 100 nM PMA, a PKC agonist, gain enhanced capability for D-Asp uptake, as shown in Fig. 5. The Figure also shows that genistein (GEN), an inhibitor of protein tyrosine kinase (PTK), induces a 40% inhibition of D-Asp uptake. The PMA-induced transport enhancement is retained when GEN is also present. The percent increment with PMA is approximately the same in the presence of GEN as in its absence, although the total uptake observed with both agents present is less than that seen with PMA alone. Inactive isomers of PMA (4α-phorbol) and GEN (daidzein) have no effect on D-Asp uptake (*data not shown*).

The effect of PMA and GEN on Na^+-dependent uptake of neutral amino acids by RBA cells is entirely different. PMA has no detectable effect on alanine uptake, but GEN causes a marked stimulation as also shown in Fig. 5. Differing effects for the two protein kinase effectors on these two Na^+-coupled transport systems shows that action of the kinases is selective for the particular transport system, and is not simply a result of a nonspecific change in cellular energy status that modifies the Na^+-gradient or membrane potential maintained by the cells. Such changes would modify capability of all Na^+-coupled transport systems in a similar way.

As little as 1 nM PMA induces detectable stimulation of D-Asp uptake, and the increase is maximal at 10 nM PMA (Fig. 6). Staurosporine (10 μM), a PKC inhibitor, totally blocks stimulation by PMA, even with phorbol concentrations as high as 1000 nM. The D-Asp concentration for the experiments shown in Figs. 5 and 6 was 100 μM. Under these conditions PMA induces about a 2-fold increase in steady-state uptake. At lower initial [D-Asp], the cells accumulate such a high percentage of available D-Asp in the absence of PMA (Table 2, Fig. 4) that only a small increment in total uptake can occur when PMA is added, even if all of the remaining extracellular amino acid is taken up. The PMA-induced increase becomes progressively more modest until only a 10–15% increase is induced at 10 μM D-Asp. However, although the change in intracellular D-Asp is relatively small, it should be noted that PMA induces a pronounced change in the steady-state extracellular [D-Asp] for these conditions, as shown in Fig. 7. At the steady-state, extracellular [D-Asp] changes by 3-fold, from about 3 μM to a little less than 1 μM. The steady-state D-Asp gradient increased to ~5,500-fold from 1,600-fold, despite the fact that intracellular [D-Asp] changed by only 10%.

Activation of D-Asp transport by PMA is not instantaneous, regardless of the initial D-Asp concentration. Table 4 shows that approximately 20 min are required after adding 100 nM PMA before the unidirectional influx of D-Asp becomes maximal. After 30 min exposure to PMA, the V_{max} for D-Asp uptake increases by about 2-fold, although the K_m remains constant, as shown in Fig. 8.

Reverse transcription was used to prepare cDNA from the mRNA fraction in preparations of total RNA from RBA. cDNA from the mRNA fraction was PCR amplified using the primers described earlier, and detected with ethidium bromide after electrophoresis on

Table 1. Uptake of 5 μM D-Asp by suspended rat brain astrocytes

	A	B	C	D	E
Incubation Time	^{3}H-D-Asp taken up	D-Asp Concentration			
min	nmoles/mg protein	Extracellular[a] (μM)	Intracellular		$\frac{ASP_{in}}{ASP_{out}}$[d]
			Calculated[b] (μM)	Measured[c] (μM)	
0	0	5.00	0	0	0
2	0.30	4.76	124	114	24
10	1.70	3.84	600	640	167
30	4.09	1.83	1,639	1,544	844
60	5.59	0.99	2,073	2,109	2,130
90	5.74	0.73	2,207	2,165	2,966
120	5.96	0.73	2,207	2,248	3,079

Each incubation vessel contained 1.68 mg cell protein in a total volume of 2.3 ml.
[a] Calculated from the CPM/ml of ^{3}H-D-Asp remaining in the supernatant after centrifugation of the cells, and the initial CPM/ml in the incubation medium.
[b] Calculated from the decrease in extracellular [D-Asp] (Column *B*) and the total cell volume in the incubation vessel (1.68 mg prot. × 2.65 μl/mg protein = 4.45 μl).
[c] Calculated from the observed amount of ^{3}H-D-Asp taken up at time *t* (Column *A*) in nmoles per mg protein, divided by the intracellular space/mg cell protein that was measured with ^{14}C-3-OMG (2.65 μl/mg protein).
[d] Column *D*/Column *B*

Table 2. Effect of monovalent ion ionophores on D-Asp uptake by rat brain astrocytes

Incubation Conditions	D-Asp Uptake[a]	
	nmoles · mg protein^{-1}	% Control
Control	6.5 ± 0.9	100
+ Oligomycin (10 μg/ml)	3.1 ± 0.7	48.0
+ Nigericin (10 μM)	2.3 ± 0.04	35.0
+ Gramicidin (10 μg/ml)	0.98 ± 0.07	15.0
+ Oligomycin, Nigericin and Gramicidin	0.08 ± 0.005	1.2

[a] Uptake is the amount D-Asp ± SEM taken up by RBA cells during a 60 min incubation interval at 37°C in 3 separate trials with different cell populations (0.8–1.0 mg cell protein per ml). The initial D-Asp concentration was 10 μM in each case.

agarose. Five independently isolated RNA preparations yielded amplification products of the expected size for GLAST and for EAAC1. No amplification products were observed for either GLT1 or EAAT4. Representative results of these five preparations along with positive controls achieved with mRNA prepared from whole rat brain are shown in Fig. 9. The GLAST and EAAC1 PCR products gave digestion products of predicted size after cutting with restriction endonucleases at unique sites followed by agarose gel electrophoresis, confirming the identity of the expected PCR products.

Preparations of total RBA protein were subjected to SDS-PAGE electrophoresis followed by Western blotting as described in the Methods section. Anti-GLAST and anti-EAAC1 antibodies detected proteins of the appropriate molecular weight (66 kDa and 69 kDa, respectively), as shown in Fig. 10. No proteins of the expected mass were detected with anti-GLT1 antibody, although all three antibodies detected their corresponding transporter subtype in protein extracts prepared from whole rat brain.

Discussion

The above data illustrate that astrocytes grown in culture can be harvested and used in suspension for characterization of the kinetic and other properties of glial Na^+-coupled glutamate transport systems. The model system provides for unidirectional influx measurements of particularly high quality because cell suspensions can be sampled rapidly and with a high degree of consistency in sample size. Multiple samples are taken for each experimental run so that measured uptake rates are established on the basis of several time points for a homogenous cell population derived from several culture plates. This technique provides more precision in the measurement of uptake rates than approaches in which cells are exposed to isotope *in situ* on a culture plate or in a culture well. In those cases only a single sample time is possible for a given plate or well, and one must assume uniformity of cell populations from one well to the next. The cell suspension model therefore offers excellent opportunity for

Table 3. Calculated K_i (K_m) values for substrates that compete for D-Asp uptake in rat brain astrocytes

Competing Substrate	Concentration μM	Initial rate of uptake of 10 μM D-Asp[a]	K_i[b]
		nmoles · min^{-1} · mg protein^{-1}	μM
None		1.00	—
L-Aspartate	50	0.245 ± 0.03	5.3
L-Aspartate	500	0.039 ± 0.002	6.6
D-Aspartate	50	0.26 ± 0.02	5.7
D-Aspartate	50	0.048 ± 0.02	8.2
L-Glutamate	50	0.56 ± 0.03	20.6
L-Glutamate	500	0.11 ± 0.006	20.0
D-Glutamate	500	0.82 ± 0.10	739.0
Serine-*O*-Sulfate	500	0.13 ± 0.03	27.4
Threo-β-hydroxyaspartate	500	0.032 ± 0.004	5.5
Kainic acid	500	0.99 ± 0.08	>3,000
Dihydrokainic acid	500	0.96 ± 0.03	>3,000

[a] The test concentration of ^{3}H-D-aspartate was 10 μM in all cases. Initial uptake was measured for a 10 min incubation interval in each case. The 'control' rate with 10 μM ^{3}H-D-aspartate, but no competing substrate (0.4 nmole · min^{-1} · mg protein^{-1}) was assigned a value of 1.0. All other rates are given as a fraction of the control rate and are mean values ± SEM for three separate experiments.

[b] K_i was calculated from the following expression, which was derived from the Michaelis-Menten equation for competitive inhibition:

$K_i = v_i \cdot K_m \cdot [I]/(v_0 - v_i)\ (K_m + [S])$

v_0 and v_i are the initial rates of D-Asp uptake for the control and 'inhibited' cases, respectively; [S] is the D-Asp concentration (10 μM); K_m is the Michaelis-Menten constant for D-Asp uptake as determined in Figure 2*B* (4.8 μM); and [I] is the concentration of the competing (inhibiting) substrate.

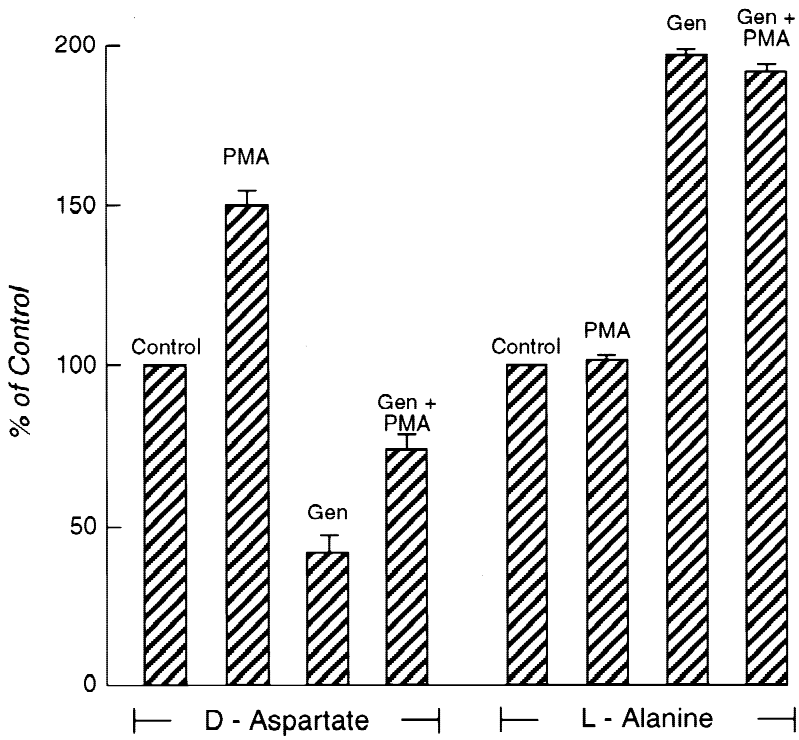

Fig. 5. Comparison of the effects of 100 nM PMA and 100 μM genistein (GEN) on uptake of ^{3}H-D-Asp or ^{3}H-L-Alanine by RBA cells. Data were normalized by setting the 'Control' uptake for each solute at 1.0 and expressing the amount of uptake observed with each test agent relative to the control rate.

assessment of the action of autocrine or paracrine factors that may modify rate or efficacy of function of these important transport events.

Short-term uptake experiments show excellent linearity of influx for 60–120 sec for a wide variety of conditions (Figs. 2 and 3). Linear uptake is maintained under control conditions for several min, although shorter measurement intervals ensure that initial uptake

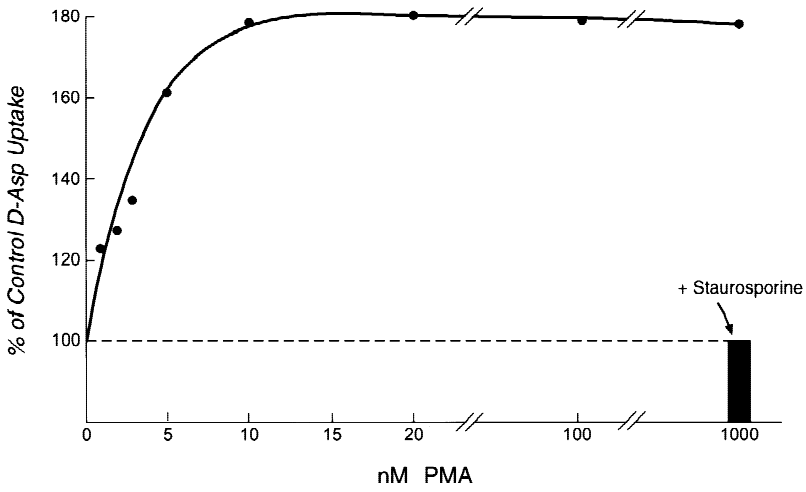

Fig. 6. Dose-response relationship between steady-state uptake of ^{3}H-D-Asp by RBA cells and PMA concentration. Cells were incubated with 100 μM D-Asp with or without PMA for a total of 80 min. The horizontal line indicates the amount of D-Asp taken up after 80 min by cells not treated with PMA. The shaded bar indicates the uptake observed when 10 μM PMA and 10 μM staurosporine were both present during the 80-min incubation interval.

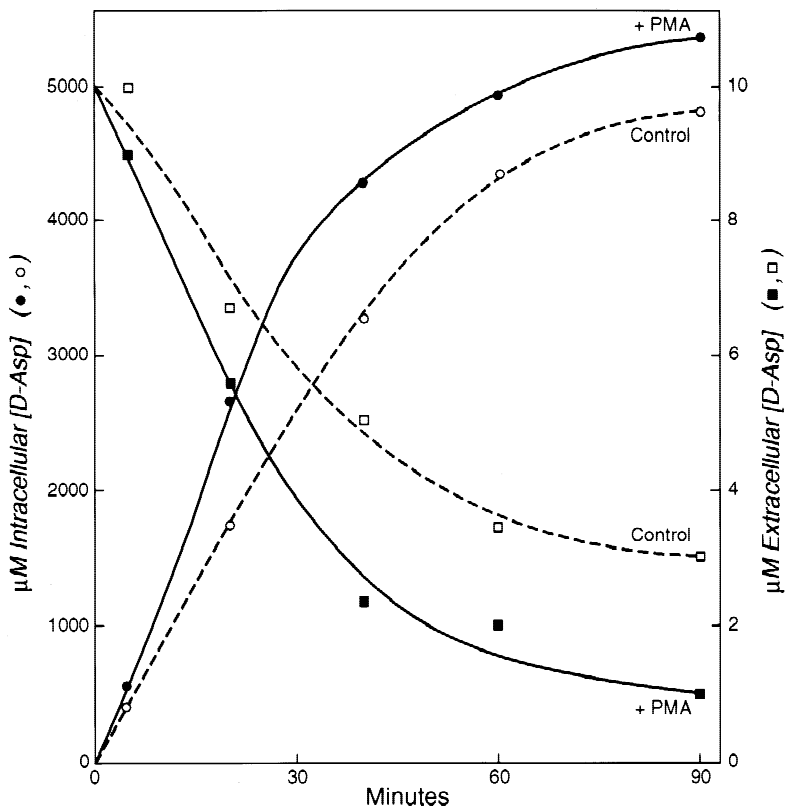

Fig. 7. Effect of 100 nM PMA on the uptake of ^{3}H-D-Asp by RBA cells and the concomitant decrease in extracellular D-Asp concentration during an 80-min incubation interval when the initial D-Asp concentration was 10 μM. The cell protein concentration was 0.8 mg/ml.

rates are indeed obtained for all conditions evaluated. Linearity of solute uptake implies that the electrochemical gradient for Na^+ maintained by the cells (i.e., Na^+ gradient and membrane potential) is stable during the uptake interval. It also implies that intracellular solute levels have not risen to the point where solute 'backflux', either on the transporter or via independent routes, begins to compromise the initial uptake rates required for useful kinetic analysis. Extrapolation of the uptake *vs.* time relationship to the y-intercept provides a correction for isotope bound to the cell surface or trapped in extracellular fluid in the cell pellets. Reliable values for these corrections are difficult to achieve with cells attached to a culture surface where isotope associated with the cells is measured for only a single time point. The amount of isotope 'trapped' under the cell syncitium can be appreciable, and any increase with time is difficult to assess. *In situ* cell cultures also can have significant unstirred water layers near the cell surface which incorporate isotope and modify the quality of data obtained in experiments where initial rates of solute uptake must be obtained to determine transport kinetics. Unstirred water

Table 4. Effect of PMA on the unidirectional influx of ^{3}H-D-Asp as a function of time after exposure of the astrocytes to the phorbol ester

Time After Addition of PMA	Rate of D-Asp Uptake nmoles · min^{-1} · mg protein^{-1}
0	1.00[a]
1	0.98
5	1.39
10	1.89
20	2.05
30	2.36
40	2.00
60	2.01

RBA cells were incubated at 37°C in the absence of D-Asp in each case prior to measurement of the D-Asp uptake rate. After 60 min, 10 μM ^{3}H-D-Asp was added, and the rate of D-Asp uptake was monitored for the following 60 sec. PMA (100 nM) was added prior to the addition of ^{3}H-D-Asp at a time appropriate for providing the indicated exposure interval to the phorbol ester.

[a] The rate of D-Asp uptake by RBA cells that were not exposed to PMA was assigned an arbitrary value of 1.0, and rates observed for the various cases in which PMA was added are expressed as a multiple of the control rate. The results shown are mean values for two trials with cell populations prepared on different days.

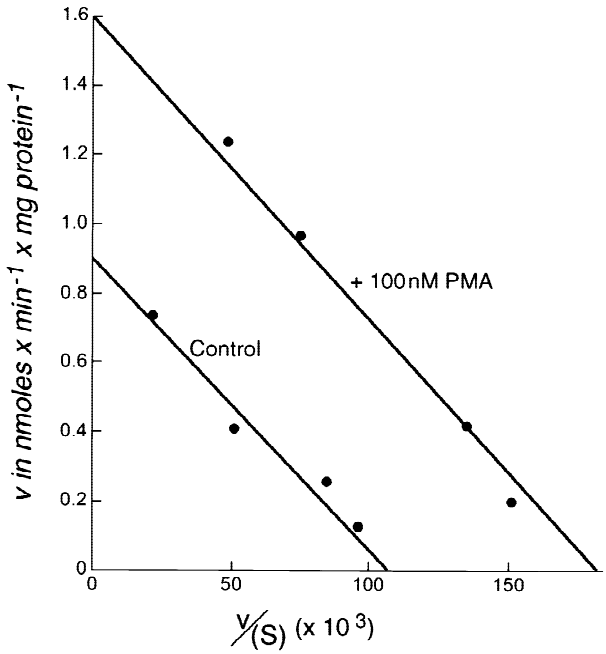

Fig. 8. Eadie-Hofstee plot showing the effect of PMA on the kinetics of D-Asp influx into RBA cells. The cells were incubated for 20 min in the absence of D-Asp either with or without 100 nM PMA prior to determining the rate of *D*-Asp uptake at 4 different aspartate concentrations. The y-intercept shows that V_{max} for uptake increased by approximately 2-fold while the K_m for D-Asp (slope) remained constant at 5 μM.

layers are small or absent in well-stirred cell suspensions, and thus offer less risk for compromising quality of kinetic data.

The magnitude of D-Asp gradients established by cultured astrocytes indicates that the harvested cells exhibit excellent retention of normal physiological properties. Steady-state synaptic concentrations of glutamate in the CNS are believed to be submicromolar (Nicholls & Atwell, 1990; Storm-Mathieson, Danbolt & Otterson, 1995). Indeed, sustained levels much above this level result in neurotoxicity leading to neuronal dysfunction and death (Choi, 1988; Rothstein et al.,1993; Schousboe & Frandsen, 1995). Transport systems capable of maintaining extracellular glutamate concentrations below 1 μM therefore are mandatory for normal CNS function. When the initial D-Asp concentration is 10 μM, 70-80% of the total aspartate in the incubation medium is taken up by cells having an intracellular volume less than 0.3% of the medium volume (Figures 4 and 7). Steady-state intracellular D-Asp concentrations reach 4–5 mM under these conditions, producing D-Asp gradients of 1,500–3,000-fold that increase to nearly 6,000-fold in the presence of PMA (Fig. 7). When the initial extracellular aspartate is 5 μM, submicromolar extracellular D-Asp concentrations are achieved even in the absence of PMA (Table 1). These values are consistent with function of a transport system that indeed can lower aspartate to the physiological levels expected for quiescent glutamatergic synapses.

The time required for D-Asp clearance to a steady-state level in the experiments described here should not be construed as related to the time for in vivo neurotransmitter clearance. For our experimental conditions, cells with a total intracellular volume of 3–6 μl cleared aspartate from a total extracellular volume of 2,300 μl. It is important to recognize that in vivo CNS intracellular and extracellular spaces are more equal in size, thus allowing much faster clearance of synaptic glutamate to low resting levels. More moderate cellular gradients would be expected than those observed for the in vitro conditions reported here. Also, under physiological conditions, metabolic conversion of glutamate to glutamine- and/or TCA-cycle intermediates enhances the cellular capability for sustaining a low extracellular glutamate concentration without the necessity for maintaining high intracellular concentrations that otherwise might limit the rate or extent of clearance.

Five different glutamate transport systems have been described for human CNS cells (Arizza et al., 1994). EAAT1 and EAAT2 (often designated GLAST and GLT1, respectively), are localized primarily in astrocytic cells (Pines et al., 1992; Storck et al., 1992). EAAT3 is the primary neuronal isoform (Velaz-Faircloth et al., 1996). It has properties similar or identical to those described for a transporter designated EAAC1 that was first described in intestinal epithelia (Kanai & Hediger, 1992; Kanai et al., 1995). EAAT4 is also a neuronal isoform, and EAAT5 is found only in retina. EAAT1, -2, and -3

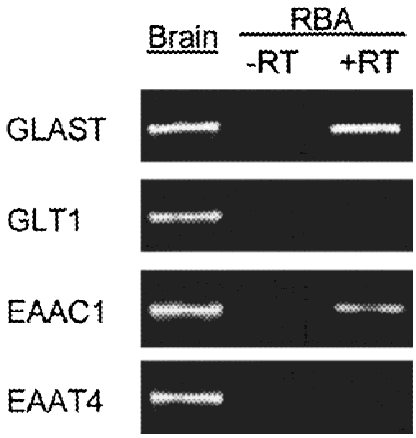

Fig. 9. RT-PCR detection of glutamate transporter mRNA in RBAs. RT was used to produce cDNA from the mRNA fraction of RNA prepared from either whole rat brain or RBA cells. Each cDNA preparation was then analyzed for GLAST, GLT1, EAAC1 and EAAT4 by PCR amplification using primers selective for each transporter subtype. Amplification products were detected after separation by agarose gel electrophoresis in ethidium bromide. All four transporter subtypes were detected in rat brain RNA (left lane, *Brain*), but only GLAST and EAAC1 in the RNA prepared from RBAs (right lane, *+RT*). No message was detected when reverse transcriptase was omitted (middle lane, *−RT*), confirming specificity of the assays. Five individual preparations of RNA from RBA cells gave results similar to those shown.

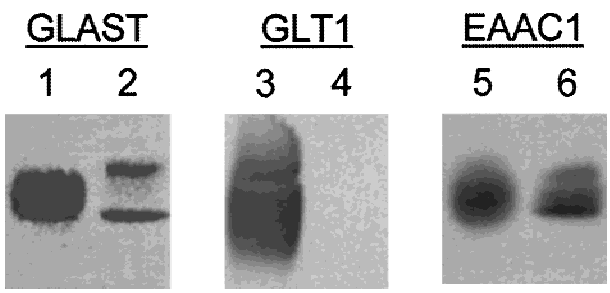

Fig. 10. Western blotting of RBA total cellular protein for glutamate transporter subtypes. Protein extraction, SDS-PAGE and Western blotting were performed as described in Methods and Materials. RBA protein (65 μg) was loaded in lanes 2, 4, and 6. Anti-GLAST and anti-EAAC1 antibodies detected proteins of ~66 kDa, and ~69 kDa, as shown. The anti-GLT1 antibody did not show immunoreactivity with any proteins of the expected molecular mass in the RBA cell protein extract. All three antibodies showed reactivity with proteins of appropriate mass in rat brain protein extracts (lanes 1, 3, and 5).

have all been studied in oocyte expression systems, and been characterized in terms of their sensitivity to various pharmacological agents. GLT1 (EAAT2) can be identified pharmacologically by its' high sensitivity to kainate (KA) and dihydrokainate (DHK) (K_i of 25–50 μM), whereas GLAST (EAAT1) and EAAC1 (EAAT3) have little or no sensitivity to either agent (K_i > 3 mM) (Arizza et al., 1994). The lack of inhibitory effect detected for 500 μM KA on uptake of 10 μM D-aspartate, and less than 5% inhibition by DHK (Table 3), points to the likelihood that GLT1 is not expressed in this cell line, a conclusion reinforced by lack of detection in Western blots. RBA D-Asp uptake is sensitive to THA and S-O-S, two agents known to inhibit both GLAST and EAAC1 (K_i 25–30 and 100–150 μM, respectively) (Arriza et al., 1994). The same authors showed that S-O-S is not a potent inhibitor of the astrocytic GLT1 transporter (K_i = 1.16 mM). Consistent with the pharmacological data, we were successful in detecting mRNA for GLAST and EAAC1, but not GLT1, using RT-PCR on RNA prepared from the cultured astrocytes. Action of a restriction enzyme after PCR amplification of the mRNA resulted in fragments consistent in size with those expected, given the primers chosen for PCR.

Thus far, there is no reliable kinetic or pharmacologic method for distinguishing between EAAC1 and GLAST. The K_m and V_{max} for these systems are nearly identical, as are K_i values for a variety of inhibitory agents (Arriza, et al., 1994). The weight of current evidence suggests that PKC activates EAAC1 (Dowd & Robinson, 1996), but inhibits GLAST (Gonzalez & Ortega, 1997; Conrad & Stoffel, 1997). If the same relative effects apply to the RBA model, the stimulation induced by PMA on RBA cells suggests that the predominant effect of the kinase is due to more pronounced changes in EAAC1 than GLAST activity. It is important to bear in mind, however, that the kinetic and regulatory properties of glutamate transporters have been observed to vary with cell type or expression system employed. Indeed, the other astrocytic isoform (GLT1), is stimulated by PMA in some cell types (Casado et al., 1993), but inhibited in others (Ganel & Crosson, 1998).

All glutamate cotransporters are known to catalyze uptake of D- and L-aspartate, and of L-glutamate, but not D-glutamate, as observed for this astrocyte model. Inhibition of D-Asp influx by alternative substrates for the transporter was used to calculate a K_i for each competing substrate. The RBA transporters have high 'affinity' for L- and D-Asp (K_i ~ 5 μM in each case) and for L-glutamate (K_i ~ 20 μM). The low K_i for L-glutamate is particularly noteworthy because it suggests retention of typical physiological substrate specificity after many in vitro passages for the cultured astrocytes. The high K_i for D-glutamate (~750 μM) indicates limited if any interaction of that D-amino acid with the transporter (Table 3). As little as 2% contamination of D-glutamate by the L isomer would give the low degree of inhibition of D-Asp uptake observed. Similar experiments reported for C6 glioma cells indicate that those cells exhibit high-affinity D-Asp uptake, but that they have lost the capability for high-affinity L-glutamate uptake (Erecinska & Silver, 1986). Recent work has shown that only the neuronal isoform (EAAC1) is expressed in C6 cells, despite the glial origin of these cells, although the lack of L-

glutamate transport implies an abnormal variant of that transporter. The RBA line used here represents another example of astrocytic expression of EAAC1, but with retention of L-glutamate transport capability. GLAST mRNA and expression is also detected, in contrast to the situation reported for C6 cells.

To our knowledge, the work described here is the first report describing GLAST expression (or mRNA) in a long-term astrocyte culture. The RBA cell line thus may be a useful model system for exploring function/regulation of either GLAST or EAAC1. The inhibitory effect induced by genistein on this system may be indicative of modulation of at least one of the two cotransporters by PTK. In some model systems, genistein has been shown to induce changes in activity of certain other plasma membrane transporters by inhibiting specific ser/thr phosphatases rather than through inhibition of PTK activity (Ilek, Fischer & Machen, 1996; Reenstra et al., 1996; Yang et al., 1997). However, we have been unable to detect any changes in RBA D-Asp transport by PKA agonists that might suggest regulatory events catalyzed by PKA ser/thr phosphorylation, or dephosphorylation at these sites. Our observations thus raise the possibility that GLAST and/or EAAC1 will prove to be modulated by PTK as well as by PKC. Pronounced activation by PMA occurs in the presence of genistein, but optimal activity is not achieved suggesting that dual phosphorylation events may be necessary for full transport capability to be achieved. However, modulation of glutamate transport by other agents that modify PTK activity has not been reported. The PMA-induced activity is inhibited by staurosporine, a potent PKC antagonist, even when PMA is present in 100-fold excess to the 10 nM optimal amount needed when staurosporine is absent (Fig. 6). No change in Na^+-coupled alanine transport was induced by PMA, showing that the effect on D-Asp transport cannot be attributed to non-specific effects of PKC on the trans-membrane electrochemical potential difference for Na^+ which provides the energy input for these transport systems. Further specificity of kinase action is indicated by the stimulatory effect of genistein on alanine transport, converse to the effect observed on D-Asp transport (Fig. 5).

Activation of glutamate transport by PMA has also been shown in primary cultures of glial cells derived from rat brain cortex (Casado et al., 1991). In that case, it was shown that transport capability was catalyzed by the kainate-sensitive GLT1 astrocytic isoform. Just as for the RBA cultured cells, optimal transport activation required approximately 30 min exposure to PMA, and resulted in a 2-fold increase in V_{max} with no detectable change in K_m. GLT1 retained responsiveness to PKC activation when expressed in *Xenopus* oocytes or in HeLa cells, and the authors showed a concomitant phosphorylation of serine 113 during transport activation. Mutant proteins lacking serine 113 retained transport capability, but no longer exhibited PMA-induced activation (Casado et al., 1993). These results, in combination with our own, suggest that both astrocytic glutamate transport systems may be activated by PKC. Both proteins have serine at position 113 or 114 in the primary sequence.

At this juncture, it is not possible to specify the physiological signal(s) that might be responsible for allowing PKC-modulated glutamate transport in astrocytes. Four of the eight PKC isoforms belonging to the so-called 'classical' PKC family are modulated by Ca^{++} ions as well as by diacylglycerol (which phorbol esters mimic). This raises the possibility that changes in intracellular Ca^{++} may induce changes in PKC activity and therefore signal modulation of transporter function. Indeed, the Ca^{++} ionophore A23187 has been shown to enhance the action of PMA on glutamate transport capability (Casado, et al., 1991).

Glutamate interaction with NMDA receptors activates an ion channel that allows Ca^{++} entry. This may play an important role in governing glutamate transport capability in cells having that receptor. Calcium ions also are known to activate phospholipase A2 that releases arachidonic acid from C#2 of various phospholipids. Because arachidonate is permeable to plasma membranes, it can escape from the cell where it is produced and interact with other cell types. Neurons and astrocytes have been shown to release arachidonate following glutamate interaction with NMDA (and perhaps other) receptors (Lazarewicz, et al., 1992, Stella, et al., 1994). Moreover, arachidonate differentially modulates function of neuronal and astrocytic glutamate transporters (Zerangue, et al., 1995). Micromolar levels of arachidonate induced a 2-fold stimulation of GLT1, but little change in EAAC1 activity, and a ~30% inhibition of transport capability by GLAST. Inhibition of specific glutamate transporters by arachidonate may be a candidate for exacerbation of glutamate-induced neurotoxicity (Rothstein et al., 1993).

It should be noted that it has also been reported that glutamate transport is 75% inhibited by PMA when GLAST is expressed in *Xenopus* oocytes or HEK293 cells (Conradt & Stoffel, 1997). Like the other PKC-mediated responses reported for GLT and GLAST, this effect also required approximately 20 min to be fully manifested. In this case, removal of all putative PKC phosphorylation sites by site-directed mutagenesis did not abolish the PMA-induced inhibition of transport. These observations suggest that there are important modulatory events for astrocytic glutamate transport mediated by PKC that do not depend solely on phosphorylation of the transport protein. This conclusion is consistent with similar ones drawn from a consideration of PKC/PKA modulation of the Na^+-coupled glucose transporter (SGLT1) where kinase-mediated changes in trans-

port capability can be demonstrated in the absence of putative phosphorylation sites on the protein (Hirsch, Loo & Wright, 1996). In that case, and for several other transporters (Bradbury & Bridges, 1994), PKC activation modifies the rate of insertion/retrieval of transport protein into or out of the plasma membrane that modulate V_{max} capability for the system. Because protein-trafficking modulatory events require time to be fully manifested, the 20–30 min interval required for PKC modulation of GLT, and that described here for GLAST/EAAC1 in the RBA line, may signify that protein trafficking events are also an important part of astrocytic glutamate transport regulation. If RBA GLAST activity proves to be inhibited by PMA, as it is in the oocyte expression system, then stimulatory changes in RBA EAAC1 activity must be particularly robust in order to more than compensate for GLAST inhibition, and allow net transport enhancement by PMA. On the other hand, if RBA GLAST activity is activated by PMA, in contrast to the expression systems so far utilized, then important questions are raised relating to influence of the host cell environment on cotransporter modulation by kinases. In either case, the RBA cell model should prove valuable for further assessment of the role which autocrine and paracrine effectors play in modulating synaptic glutamate/aspartate clearance.

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References

Amara, S.G., Kuhar, M.J. 1993. Neurotransmitter transporters. *Ann. Rev. Neurosci.* **16:**73–93

Arriza, J.L., Fairman, W.A., Wadiche, J.I., Murdoch, G.H., Kavanaugh, M.P., Amara, S.G. 1994. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.* **14:**5559–5569

Bradbury, N.A., Bridges, R.J. 1994. Role of membrane trafficking in plasma membrane solute transport. *Am. J. Physio.* **267:**C1–C24

Casado, M., Zafra, F., Aragon, C., Gimenez, C. 1991. Activation of high-affinity uptake of glutamate by phorbol esters in primary glial cell cultures. *J. Neurochem.* **57:**1185–1190

Casado, M., Bendahan, A., Zafra, F., Danbolt, N.C., Aragon, C., Gimenez, C., Kanner, B.I. 1993. Phosphorylation and modulation of brain glutamate transporters by protein kinase C. *J. Biol. Chem.* **268:**27,313–27,317

Choi, D.W. 1988. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1:**623–634

Conradt, M., Stoffel, W. 1997. Inhibition of the high-affinity brain glutamate transporter GLAST-1 via direct phosphorylation. *J. Neurochem.* **68:**1244–1251

Csaky, T., Thale, M. 1960. Effect of ionic environment on intestinal sugar transport. *J. Physiol.* **151:**59–65

Dowd, L.A., Robinson, M.B. 1996. Rapid stimulation of EAAC1-mediated Na^+-dependent L-Glutamate transport activity in C6 glioma cells by phorbol ester. *J. Neurochem.* **67:**508–516

Erecinska, M., Silver, I.A. 1986. The role of glial cells in regulation of neurotransmitter amino acids in the external environment. I. Transmembrane electrical and ion gradients and energy parameters in cultured glial-derived cell lines. *Brain Research* **369:**193–202

Erecinska, M., Troeger, M.B., Wilson, D.F., Silver, I.A. 1986. The role of glial cells in regulation of neurotransmitter amino acids in the external environment. II. Mechanism of aspartate transport. *Brain Research* **369:**203–214

Flott, B., Seifert, W. 1991. Characterization of glutamate uptake systems in astrocyte primary cultures from rat brain. *Glia* **4:**365–402

Ganel, R., Crosson, C.E. 1997. Modulation of human glutamate transporter activity by phorbol ester. *J. Neurochem.* **70:**993–1000

Gonzalez, M.I., Ortega, A. 1997. Regulation of the Na^+-dependent high affinity glutamate/aspartate transporter in cultured Bergmann glia by phorbol esters. *J. Neurosci. Res.* **50:**585–590

Hertz, L., Schousboe, A. 1986. Role of astrocytes in compartmentation of amino acid and energy metabolism. *In:* Astrocytes—Biochemistry, Physiology, and Pharmacology of Astrocytes, Vol. 2, S. Fedoroff, A. Vernadakis, editors. pp. 179–208. Academic Press, New York

Hirsch, J.R., D.D.F. Loo and E.M. Wright (1996) Regulation of Na^+/glucose cotransporter expression by protein kinases in *Xenopus laevis* oocytes. J. Biol. Chem. **271:**14,740–14,746

Ilek, B., Fischer, H., Machen, T.E. 1996. Alternate stimulation of apical CFTR by genistein in epithelia. *Am. J. Physiol.* **270:**C265–C275

Kanai, Y., Hediger, M.A. 1992. Primary structure and functional characterization of a high affinity glutamate transporter. *Nature* **360:**467–471

Kanai, Y., Nussberger, S., Romero, M.F., Boron, W.F., Hebert, S.C., Hediger, M.A. 1995. Electrogenic properties of the epithelial and neuronal high affinity glutamate transporter. *J. Biol. Chem.* **270:**16,561–16,568

Kaufman, E., Driscoll, B.F. 1992. Carbon dioxide fixation in neuronal and astroglial cells in culture. *J. Neurochem.* **58:**258–262

Kimmich, G.A. 1975. Preparation and characterization of isolated intestinal epithelial cells and their use in studying intestinal transport. *In:* Methods in Membrane Biology. E.D. Korn, editor. pp 51–111. Plenum Press, New York

Kimmich, G.A. 1990. Membrane potentials and the mechanism of intestinal Na^+-dependent sugar transport. *J. Membrane Biol.* **114:**1–27

Kimmich, G.A., Randles, J. 1984. The Na^+:sugar coupling stoichiometry in isolated intestinal epithelial cells. *Am. J. Physiol.* **247:**C74–C82

Kimmich, G.A., Randles, J., Wilson, J. 1994. Na^+-coupled alanine transport in LLC-PK1 cells. *Am. J. Physiol.* **267:**C1119–C1129

Klockner, U., Storck, T., Conradt, M., Stoffel, W. 1993. Electrogenic L-glutamate uptake in Xenopus laevis expressing a cloned rat brain L-glutamate/L-aspartate transporter (GLAST1). *J. Biol. Chem.* **268:**14,594–14,596

Kondo, K., Hashimoto, H., Kitanaka, J., Sawada, M., Suzumura, A., Marunouchi, T., Baba, A. 1995. Expression of glutamate transporters in cultured glial cells. *Neurosci. Lett.* **188:**140–142

Lazarewicz, J.W., Salinska, E., Wroblwski, J.T. 1992. NMDA receptor-mediated arachidonic acid release in neurons: Role in signal transduction and pathological aspects. *Adv. Exp. Med. Biol.* **318:**73–89

Lehre, K.P., Levy, L.M., Ottersen, O.P., Storm-Mathisen, J., Danbolt, N.C. 1995. Differential expression of two glial glutamate transporters in rat brain: Quantitative and immunocytochemical observations. *J. Neurosci.* **15:**1835–1853

Lester, H.A., Mager, S., Quick, M.W., Corey, J.L. 1994. Permeation properties of neurotransmitter transporters. *Ann. Rev. Pharmacol. Toxicol.* **34:**219–249

Lowry, O.H., Rosebrough, M.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Nicholls, D.G., Atwell, D. 1990. The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* **11:**462–466

Peng, T., Sheu, S-S. 1994. Arachidonic acid induced increase of intracellular sodium and calcium concentration in astrocytes: Underlying mechanisms and functional significance. pp. 128–132. Doctoral Dissertation collection, Miner Library, Sch. Med. and Dent., Univ. of Rochester

Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., Kanner, B.I. 1992. Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360:**464–467

Randles, J., Kimmich, G.A. 1978. Effects of phloretin and theophylline on 3-O-methylglucose transport by intestinal epithelial cells. *Am. J. Physiol.* **234:**C64–C72

Reenstra, W.W., Yurko-Mauro, K., Dam, A., Raman, S., Shorten, S. 1996. CFTR chloride channel activation by genistein: the role of serine/threonine protein phosphatases. *Am. J. Physiol.* **271:**C650–C657

Restrepo, D., Kimmich, G.A. 1985a. Kinetic analysis of the mechanism of intestinal Na^+-dependent sugar transport. *Am. J. Physiol.* **248:**C498–C509

Restrepo, D., Kimmich, G.A. 1985b. The mechanistic nature of the membrane potential dependence of sodium-sugar cotransport in small intestine. *J. Membr. Biol.* **87:**159–172

Rothstein, J.D., Jin, L., Dykes-Hoberg, M., Kuncl, R.W. 1993. Chronic glutamate uptake inhibition produces a model of slow neurotoxicity. *Proc. Natl. Acad. Sci. USA* **90:**6591–6595

Rothstein, J.D., Martin, L., Levey, A.I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., Kuncl, R.W. 1994. Localization of neuronal and glial glutamate transporters. *Neuron* **13:**713–725

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P., Welty, D.F. 1996. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16:**675–686

Shank, R.P., Bennett, G.S., Freytag, S.O., Campbell, GL-M. 1985. Pyruvate carboxylase: an astrocyte specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Res.* **329:**364–367

Schousboe, A., Frandsen, A. 1995. Glutamate receptors and neurotoxicity. In: CNS Neurotransmitters and Neuromodulators: Glutamate. T.W. Stone, editor. pp. 239–251. CRC Press, New York

Stella, N., Tence, M., Glowinski, J., Premont, J. 1994. Glutamate-evoked release of arachidonic acid from mouse brain astrocytes. *J. Neurosci.* **14:**568–575

Storck, T., Schulte, S., Hoffman, K., Stoffel, W. 1992. Structure, expression, and functional analysis of a Na^+-dependent glutamate/aspartate transporter from rat brain. *Proc. Nat. Acad. Sci. USA* **89:**10,955–10,959

Storm-Mathiesen, J., Danbolt, N.C., Ottersen, O.P. 1995. Localization of glutamate and its membrane transport proteins. *In:* CNS Neurotransmitters and Neuromodulators: Glutamate. T.W. Stone, editor. pp. 1–18. CRC Press, New York

Velaz-Faircloth, M., McGraw, T.S., Malandro, M.S., Fremeau, R.T., Kilberg, M.S., Anderson, K.J. 1996. Characterization and distribution of the neuronal glutamate transporter EAAC1 in rat brain. *Am. J. Physiol.* **270:**C67–C75

Waniewski, R.A., Martin, D.L. 1986. Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. *J. Neurosci.* **47:**304–313

Wilson, J., Kimmich, G.A. 1996. A model for the kinetic mechanism of sodium-coupled L-alanine transport in LLC-PK1 cells. *Am. J. Physiol.* **270:**C49–C56

Wingrove, T.G., Kimmich, G.A. 1987. High affinity L-aspartate transport in chick small intestine. *Am. J. Physiol.* **252:**C105–C114

Wingrove, T.G., Kimmich, G.A. 1988. Low affinity L-aspartate transport with 2:1 coupling stoichiometry for Na^+/Asp^-. *Am. J. Physiol.* **255:**C737–C744

Yang, I.C.-H., T.-H. Huang, F. Wang, E.M. Price, Hwang, T-C. 1997. Modulation of CFTR chloride channels by calyculin A and genistein. *Am. J. Physiol.* **272:**C142–C155

Zerangue, N., Arriza, J.L., Amara, S.G., Kavanaugh, M.P. 1995. Differential modulation of human glutamate transporter subtypes by arachidonic acid. *J. Biol. Chem.* **270:**6433–6435