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Differentiation of Substrate and Nonsubstrate Inhibitors of the High-Affinity, Sodium-Dependent Glutamate Transporters[1]

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ABSTRACT

Within the mammalian central nervous system, the efficient removal of L-glutamate from the extracellular space by excitatory amino acid transporters (EAATs) has been postulated to contribute to signal termination, the recycling of transmitter, and the maintenance of L-glutamate at concentrations below those that are excitotoxic. The development of potent and selective inhibitors of the EAATs has contributed greatly to the understanding of the functional roles of these transporters. In the present study, we use a library of conformationally constrained glutamate analogs to address two key issues: the differentiation of substrates from nontransportable inhibitors and the comparison of the pharmacological profile of synaptosomal uptake with those of the individual EAAT clones. We demonstrate that the process of transporter-mediated heteroexchange can be exploited in synaptosomes to rapidly distinguish transportable from nontransportable inhibitors. Using this approach, we demonstrate that 2,4-methanopyrrolidine-2,4-dicarboxylate, *cis*-1-aminocyclobutane-1,3-dicarboxylate, and L-*trans*-2,4-pyrrolidine dicarboxylate act as substrates for the rat forebrain synaptosomal glutamate uptake system. In contrast, L-*anti-endo*-3,4-methanopyrrolidine-3,4-dicarboxylate, L-*trans*-2,3-pyrrolidine dicarboxylate, and dihydrokainate proved to be competitive inhibitors of D-[^{3}H]aspartate uptake that exhibited little or no activity as substrates. When these same compounds were characterized for substrate activity by recording currents in voltage-clamped *Xenopus laevis* oocytes expressing the human transporter clones EAAT1, EAAT2, or EAAT3, it was found that the pharmacological profile of the synaptosomal system exhibited the greatest similarity with the EAAT2 subtype, a transporter believed to be expressed primarily on glial cells.

The ability of L-glutamate to act within the mammalian central nervous system (CNS) as both the predominant excitatory neurotransmitter and as a potent neurotoxin suggests that its extracellular concentration must be carefully regulated. On one hand, the activation of excitatory amino acid (EAA) receptors by L-glutamate is critical to both standard fast synaptic transmission and the higher order signaling required in development, learning, and memory (for review, see Cotman et al., 1995). On the other hand, overactivation of the EAA ionotropic receptors can induce excitotoxic neuronal injury, a pathological mechanism found to be involved in a wide variety of CNS disorders (e.g., stroke, trauma, amyotrophic lateral sclerosis, Huntington's disease) (Choi, 1994, 1995; Rothman and Olney, 1995; Leigh and Meldrum, 1996; Arzberger et al., 1997; Olney et al., 1997). The balance between these physiological and pathological actions of L-glutamate is thought, at least in part, to be kept in check by the rapid removal of this acidic amino acid from the synaptic cleft. Although a number of systems have been identified that are capable of transporting L-glutamate into cells, the majority of uptake within the CNS seems to be mediated by the high-affinity, sodium-dependent excitatory amino acid transporters (EAATs) (for review, see Gegelashvili and Schousboe, 1997; Vandenberg, 1998). Transport of L-glutamate by the EAATs is electrogenic and is driven primarily by the transmembrane sodium gradient generated by Na^+/K^+ ATPases. The efficient removal of L-glutamate from the extracellular space by these systems has been postulated to contribute to signal termination, the recycling of the transmitter, and the maintenance of L-glutamate at concentrations below those which are excitotoxic (for review, see Takahashi et al., 1997).

Several subtypes of sodium-dependent glutamate trans-

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ABBREVIATIONS: CNS, central nervous system; EAA, excitatory amino acid; EAAT, excitatory amino acid transporter; PDC, pyrrolidine dicarboxylate; KA, kainate; DHK, dihydrokainate; ACBD, 1-aminocyclobutane-1,3-dicarboxylate; MPDC, methano-pyrrolidine-2,4-dicarboxylate; THA, *threo*-hydroxyaspartate; ADC, azetidine-2,3-dicarboxylate; *cis*-5-Me-L-*trans*-2,3-PDC, (2*S*,3*S*,5*S*)-5-methyl-pyrrolidine dicarboxylate.

porters have been isolated and cloned from mammalian tissue, including GLAST and GLT-1 from rat brain and EAAC-1 from rabbit intestine (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). The human homologs of these transporters, EAAT1, EAAT2, and EAAT3, respectively, have also been identified (Arriza et al., 1994). Studies of cellular expression indicate that EAAT1/GLAST and EAAT2/GLT-1 are present primarily on astrocytes, whereas the EAAT3/EAAC-1 subtype is preferentially found on neurons (for review, see Gegelashvili and Schousboe, 1998). More recent investigations have revealed two additional human subtypes: EAAT4, which is selectively found in cerebellar tissue, and EAAT5, a retinal transporter (Fairman et al., 1995; Arriza et al., 1997). Cloning of the transporters provided the means with which to individually express the proteins in cell systems, such as *Xenopus laevis* oocytes, hence allowing for the delineation of more precise functional characteristics. Current modeling of EAAT stoichiometry suggests that L-glutamate is cotransported with three sodium ions and a proton in exchange for one potassium ion, and that uptake is also associated with a nonthermodynamically coupled chloride conductance (Zerangue and Kavanaugh, 1996; Levy et al., 1998).

Our understanding of the pharmacological specificity of these transporters, as well as their functional roles, has benefited greatly from the development of potent and selective inhibitors. Conformationally constrained analogs of L-glutamate, like those that proved invaluable in the delineation of the EAA receptors, have been especially useful in this regard (Chamberlin et al., 1998). As a consequence of their restricted flexibility, the compounds are capable of mimicking only a very limited number of the conformations that are attainable by L-glutamate. Not only does this allow for the positions of the functional groups required for binding to the systems to be defined, it also decreases the number of binding sites the compound can occupy, thereby increasing selectivity. Often these compounds are designed with the goal of embedding L-glutamate or L-aspartate (or a portion thereof) into a ring system. This strategy led to the development of uptake inhibitors such as L-*trans*-2,4-pyrrolidine dicarboxylate (L-*trans*-2,4-PDC), (2*S*,3*S*,4*R*)-2-(carboxycyclopropyl)glycine (-CCGIII), *cis*-1-aminocyclobutane-1,3-dicarboxylate (*cis*-ACBD), and L-*anti-endo*-3,4-methanopyrrolidine-3,4-dicarboxylate (L-*anti-endo*-3,4-MPDC). Initially used to characterize the pharmacology of glutamate uptake in physiological preparations such as synaptosomes, tissue slices, and cell cultures, these inhibitors have more recently been employed in expression systems and used to probe the specificity of the cloned transporters (Arriza et al., 1994; Yamashita et al., 1995; Esslinger et al., 1998).

In the present work, a library of conformationally constrained glutamate analogs (Fig. 1) is brought to bear upon the rat forebrain synaptosomal glutamate transport systems for the purpose of addressing two key issues: the molecular differentiation of substrate and nonsubstrate inhibitors and the comparison of this extensively used physiological preparation to the activities of the cloned glutamate transporters. We demonstrate that the process of transporter-mediated heteroexchange, in which an externally applied substrate stimulates the efflux of an internal (radiolabeled) substrate

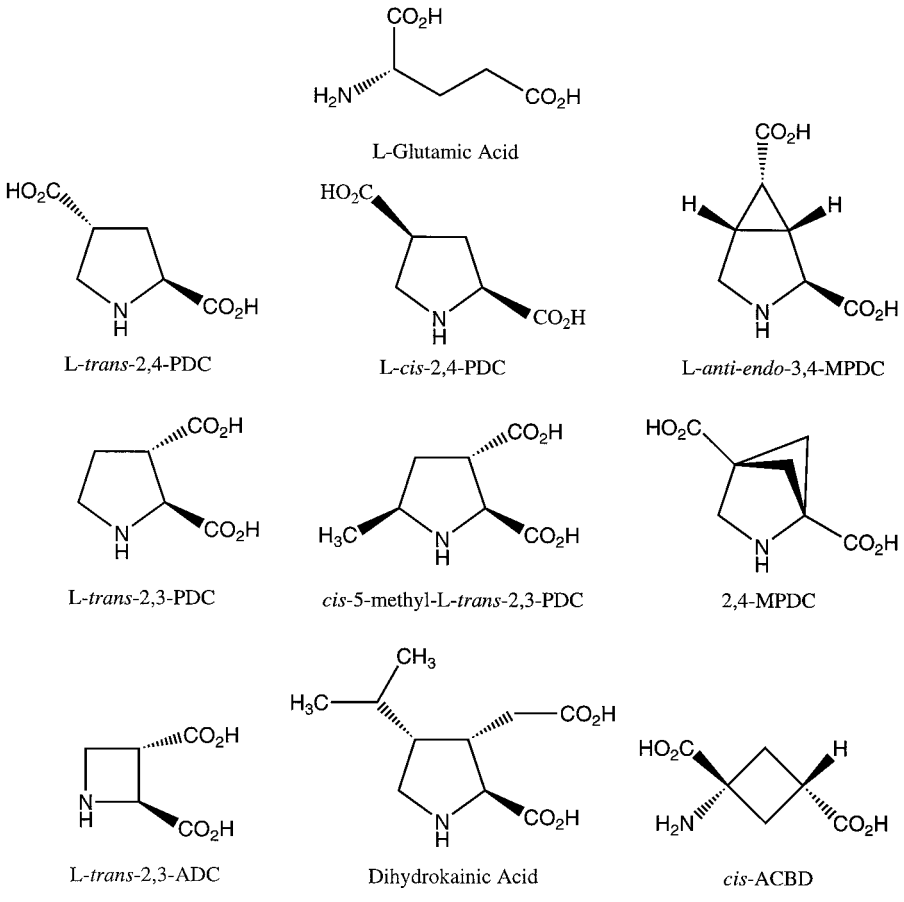

Fig. 1. Conformationally constrained inhibitors of sodium-dependent, high-affinity L-glutamate transporters.

present in the synaptosomes, can be exploited to rapidly differentiate substrates from nontransportable inhibitors. A number of potent inhibitors were identified that shared similar positionings of their functional groups, yet exhibited markedly different capacities to serve as substrates for the transporter. These analogs were then used in molecular modeling studies to refine our pharmacophore model of the substrate-binding site on the transporter. Interestingly, the resulting pharmacological profile of substrates and nontransportable inhibitors also demonstrated that the synaptosomal preparation exhibited the greatest correspondence with EAAT2, a transporter believed to be primarily of glial origin (for review, see Gegelashvili and Schousboe, 1997).

Experimental Procedures

Materials. D-[^{3}H]Aspartate was purchased from NEN (Boston, MA). L-Glutamate, D-aspartate, dihydrokainate (DHK), kainate (KA), and DL-β-THA were obtained from Sigma (St. Louis, MO). *cis*-ACBD was purchased from Tocris (Ballwin, MO). 2,4-MPDC, L-*cis*-2,4-PDC, L-*trans*-2,4-PDC, L-*trans*-2,3-PDC, (2*S*,3*S*,5*S*)-5-methylpyrrolidine-2,3-dicarboxylate, L-*anti-endo*-3,4-MPDC, and L-*trans*-2,3-ADC were synthesized as described (Bridges et al., 1991, 1993, 1994; Humphrey et al., 1994; Willis et al., 1997; Esslinger et al., 1998).

Synaptosomal Preparation and Transport. Rat cortical synaptosomes were prepared essentially by the procedure of Booth and Clark (1978), using a discontinuous Ficoll/sucrose gradient as described previously (Bridges et al., 1991). Uptake of D-[^{3}H]aspartate was measured essentially by the method of Kuhar and Zarbin (1978). Synaptosomes were suspended in assay buffer (10 mM Tris-acetate, 128 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4) at a concentration of 0.2 mg of protein/ml. After a 5-min preincubation at 25°C, uptake assays were initiated by the addition of D-[^{3}H]aspartate (1–20 μM) to the synaptosomes. In the inhibition experiments, D-[^{3}H]aspartate and inhibitor were added simultaneously. After a 2-min incubation at 25°C, the assay was rapidly quenched by the addition of 4 ml of ice-cold assay buffer. The suspension was quickly filtered through Whatman GF/F micro-fiber filters and rinsed with an additional 4 ml of ice-cold assay buffer. Filters were transferred to scintillation fluid (National Diagnostics, Atlanta, GA) and the retained radioactivity was quantified by liquid scintillation counting. Within each experiment, uptake rates were determined in duplicate. Nonspecific uptake and/or binding was corrected for by subtracting the amount of D-[^{3}H]aspartate accumulated at 4°C. Previous studies demonstrated that under these conditions, uptake was linear with respect to both time and protein content (data not shown). Protein levels were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) (Smith et al., 1985).

Determination of Substrate Activity. Heteroexchange-mediated release of D-[^{3}H]aspartate from synaptosomes was quantified as described by Chamberlin et al. (1998). Essentially, synaptosomes were suspended in assay buffer at a concentration of 0.45 mg of protein/ml. Aliquots (10 ml) of this suspension were allowed to incubate with either 2.5 μM D-[^{3}H]aspartate or 5.0 μM [^{3}H]γ-aminobutyric acid (GABA; NEN; Boston, MA) for 15 min at 25°C. Synaptosomes containing the ^{3}H-substrates were reisolated by centrifugation (28,150*g*, 20 min, 4°C), rinsed, resuspended to 1 mg of protein/ml of ice-cold assay buffer, and maintained on ice. The total content of radiolabel in the synaptosomes was determined by adding 100 μL of the suspension to 2.9 ml of ice-cold assay buffer and vacuum filtering as described above. Radioactivity present in the synaptosomes was quantified by liquid scintillation counting. This value was determined at the beginning, middle, and end of each experiment to ensure that the synaptosomal content of either D-[^{3}H]aspartate or [^{3}H]GABA did not change during maintenance on ice and that different preparations contained similar levels of each of the radiolabeled substrates. Furthermore, because GABA (unlike D-aspartate) could potentially be metabolized by the synaptosomal preparation, the radioactivity present in the synaptosomes after reisolation and incubation on ice (40 min) was evaluated by thin-layer chromatography. The synaptosomes containing the [^{3}H]GABA were lysed in 5% sulfosalicylic acid, centrifuged, and aliquots spotted on plastic-backed cellulose thin-layer chromatography plates in combination with standard GABA as a carrier. Two different solvent systems were used: phenol (lower phase of a mixture of phenol and water) and *tert*-butyl alcohol, methylethyl-ketone, water, ammonium hydroxide; 40:30:20:10) (Fink et al., 1963). Recovery was determined by ninhydrin staining and comparison with standard [^{3}H]GABA chromatographed in a parallel lane. The radioactivity still present as GABA after the reisolation and a 40-min incubation on ice were calculated to be 84 ± 7% and 75 ± 13% (mean ± S.D., n = 3) in the phenol (R_f 0.8 ± 0.03) and ammonia/*tert*-butanol phenol (R_f 0.25 ± 0.03) solvent systems, respectively.

Assays quantifying the efflux of D-[^{3}H]aspartate or [^{3}H]GABA were initiated by adding a 100-μL aliquot of synaptosomes containing the radiolabeled compounds to 2.9 ml of assay buffer pre-equilibrated and maintained at 37°C. In those experiments in which heteroexchange was examined, the indicated compounds were included in the assay buffer. Assays were terminated 1 to 5 min later by the addition of ice-cold assay buffer. The radioactivity remaining in the synaptosomes was determined as described above.

Oocyte Transport. Capped RNA was transcribed from linearized plasmids containing the coding regions of EAAT1–3 (Arriza et al., 1994). RNA (50 ng) was injected into stage V *X. laevis* oocytes and experiments were performed 2 to 6 days later. Current recordings were made with a two-microelectrode voltage clamp circuit. Electrodes contained 3 M KCl and had resistances of 100 to 500 kΩ. Oocytes were voltage-clamped and continuously superfused with Ringer recording solution containing: 98.5 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 5 mM HEPES, pH 7.5. Glutamate and analogs were bath-applied by switching to a solution containing the compounds at the indicated concentrations. The concentration-dependence of the currents induced by the compounds were fitted by least-squares to the equation $I = I_{max}([compound]/([compound] + K_m)$. K_m values are expressed as mean ± S.E. from fits to individual oocytes. Nontransportable inhibitors were coapplied with varying concentrations of L-glutamate, and Schild analysis (Arunlakshana and Schild, 1959) was performed to obtain estimates of inhibitor equilibrium dissociation constants.

Results

Competitive Inhibition of D-[^{3}H]Aspartate Uptake into Rat Forebrain Synaptosomes. The need to kinetically characterize the concentration-dependence with which the analogs inhibited the synaptosomal uptake of D-[^{3}H]aspartate uptake was 2-fold. It was necessary to 1) confirm that the mechanism of inhibition was competitive and 2) determine K_i values so that similar levels of binding site occupancy could be maintained in the exchange studies. In addition to those conformationally constrained pyrrolidine dicarboxylates previously identified as competitive inhibitors, this series of compounds included: L-*cis*-2,4-PDC, L-*trans*-2,3-ADC, (2*S*,3*S*,5*S*)-5-methyl-PDC (*cis*-5-Me-L-*trans*-2,3-PDC), and 2,4-MPDC (Fig. 1). A representative Lineweaver-Burk plot and replot of $K_{m,app}$ versus inhibitor concentration (Fig. 2, inset) for 2,4-MPDC are depicted in Fig. 2. The pattern of inhibition observed in this analysis, as well for those of all of the other analogs, was consistent with competitive inhibition. The resulting K_i values, reported as the mean ± S.E. from at least three such analyses, are reported in Table 1. On the basis of these K_i values, the analogs could be divided be-

tween those that were similar in potency to L-glutamate and L-aspartate (2–5 μM; e.g., L-*trans*-2,4-PDC, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC) and those more similar to the well known inhibitor DHK (30 μM; e.g., L-*trans*-2,3-PDC, *cis*-5-Me-L-*trans*-2,3-PDC, and *cis*-ACBD. Two of the inhibitors, L-*cis*-2,4-PDC and L-*trans*-2,3-ADC, also proved to be competitive inhibitors, but were markedly less potent than DHK, exhibiting K_i values of about 70 μM.

Exploiting the Process of Heteroexchange to Characterize Substrate Activity. The conformationally constrained competitive inhibitors were then tested as potential substrates by assaying their ability to exchange with, and stimulate the efflux of, a radiolabeled substrate from inside the synaptosomes. Thus, synaptosomes were incubated with D-[^{3}H]aspartate, reisolated by ultracentrifugation, resuspended (D-[^{3}H]aspartate content = 1152 ± 65 pmol/mg protein) and then diluted 30-fold into assay buffer at 37°C in the presence and absence of potential substrates. Analogs were tested at 100 μM and, to ensure comparable levels of occupancy of the transporter binding sites, at a concentration approximately 10-fold greater than the K_i values with which they inhibited uptake. The extent of efflux was quantified over a 2-min period. The values were corrected for the efflux that occurred in the absence of inhibitor (228 ± 8 pmol/mg protein/2 min) and are summarized in Table 1. The analogs exhibited a wide range of substrate activities that did not necessarily correlate with the ability of the compounds to bind to the transporter. For example, L-glutamate and L-*anti-endo*-3,4-MPDC were equally effective at inhibiting uptake (K_i values ≈ 5 μM), although L-*anti-endo*-3,4-MPDC exhibited only a third of the substrate activity of L-glutamate. In contrast, 2,4-MPDC (K_i = 6.8 μM) proved to be almost equipotent with L-glutamate in stimulating exchange, indicating that conformational restriction does not necessarily convey a low substrate activity. Interestingly, *cis*-ACBD and L-*cis*-2,4-PDC (K_i = 30 μM and 66 μM, respectively) also produced a marked increase in the efflux of D-[^{3}H]aspartate when present at concentrations sufficient to ensure high levels of binding. These results suggest that even moderate-to-weak inhibitors can be effectively translocated once bound to the transporter substrate site. Although KA, DHK, L-*trans*-2,3-PDC, and *cis*-5-Me-L-*trans*-2,3-PDC were identified as competitive inhibitors, these compounds did not produce levels of efflux that were significantly different from what was observed in the absence of inhibitor. Additionally, when included in the efflux assay in combination with L-glutamate, both DHK and L-*trans*-2,3-PDC effectively attenuated glutamate-mediated exchange, consistent with the action of nontransportable inhibitors. Similar results were observed in more detailed studies that examined the time course with which the substrates and nonsubstrate inhibitors altered the efflux of D-[^{3}H]aspartate from the synaptosomes. As illustrated in Fig. 3A, little or no efflux of radiolabel was observed when the synaptosomes were maintained at 4°C (≤ 40 min). However, when the synaptosomes were diluted into assay buffer at 37°C in the presence of L-glutamate (10 μM), the resulting efflux of D-[^{3}H]aspartate was more than 2-fold greater than that observed in assay buffer alone. On the other hand, L-*trans*-2,3-PDC (300 μM) not only failed to stimulate heteroexchange by itself, but it also reduced the level of glutamate-mediated heteroexchange when it was combined in the assay with this substrate (Fig. 3A).

The specificity of the exchange process, which is critical to evaluating the utility of this approach for identifying gluta-

TABLE 1

K_i values and exchange rates for transport inhibitors

K_i values ± S.D. reported in this table represent the average of at least three separate Lineweaver-Burk analyses that were carried out as described in Figure 2. The synaptosomal efflux of D-[^{3}H]aspartate induced by the inhibitors was quantified over a 2-min period at 37°C and was corrected for the efflux that occurred in the absence of added compound (228 ± 8 pmol/mg protein/2 min). Inhibitors were tested both at 100 μM and, to ensure comparable levels of transporter binding, at concentrations approximately 10 times greater than the K_i values for which they inhibited D-[^{3}H]aspartate uptake. Values represent mean ± S.E. from at least four separate determinations conducted in duplicate.

Compound	K_i	Concentration	Exchange rate (pmol/mg protein/2 min)
		μM	
L-Glutamate	4.9 ± 1	100	362 ± 12
		50	344 ± 16
		10	316 ± 22
2,4-MPDC	6.8 ± 3	100	282 ± 28
		75	280 ± 30
L-Aspartate	1.7 ± 0.6	100	278 ± 16
		20	266 ± 22
cis-ACBD	30 ± 2	100	266 ± 4
		300	270 ± 8
L-*cis*-2,4-PDC	66 ± 6	100	206 ± 16
		500	260 ± 22
L-*trans*-2,4-PDC	1.5 ± 0.5	100	250 ± 28
		15	208 ± 28
L-β-THA	2.0 ± 1	100	172 ± 24
		20	162 ± 16
L-*trans*-2,3-ADC	78 ± 6	100	146 ± 14
		800	156 ± 26
L-*anti-endo*-3,4-MPDC	4.9 ± 2	100	120 ± 20
		50	120 ± 18
Kainate	59 ± 6	100	0 ± 1.5
		600	0 ± 0
cis-5-Me-L-*trans*-2,3-PDC	37 ± 6	100	3 ± 6
		350	0 ± 0
DHK	28 ± 2	100	0 ± 3
		300	11.4 ± 6
DHK+ L-Glutamate		300 10	60 ± 12
L-*trans*-2,3-PDC	33 ± 6	100	3 ± 6
		300	19 ± 6
L-*trans*-2,3-PDC + L-Glutamate		300 10	76 ± 16

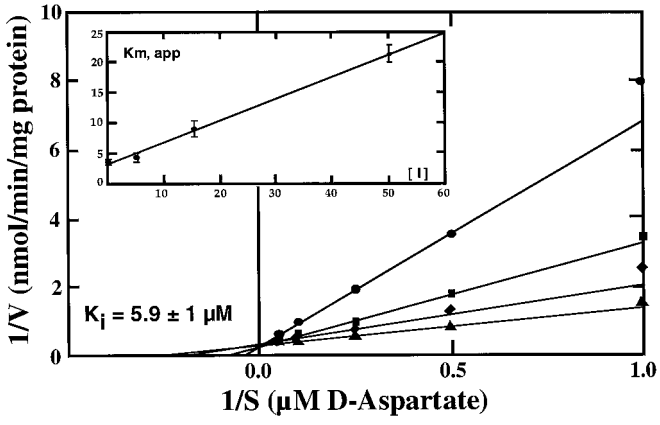

Fig. 2. Representative Lineweaver-Burk plot demonstrating the competitive inhibition of D-[^{3}H]aspartate uptake into forebrain synaptosomes by 2,4-MPDC. The inset shows a replot of $K_{m,app}$ versus [2,4-MPDC]. The plots shown were generated using the K-cat kinetic program (BioMetallics Inc., Princeton, NJ) with weighting based on constant relative error, and yielded values of: K_m = 3.8 μM (D-aspartate), K_i = 9.1 μM, and a V_{max} of 3.3 nmol/min/mg protein. Eight such analyses yielded an average K_i = 6.8 ± 3 μM.

mate transporter substrates and inhibitors, was evaluated by conducting a parallel series of experiments with GABA. As shown in Fig. 3A, the efflux of D-[^{3}H]aspartate was unaffected by the inclusion of GABA (50 μM) in the dilution buffer. Conversely (Fig. 3B), the efflux of radiolabel from synaptosomes containing [^{3}H]GABA (3887 $\pm$ 263 pmol/mg protein) was markedly enhanced by the presence of GABA (50 μM; i.e., $\approx 10 \times K_i$) in the assay buffer, but not by L-glutamate (10 μM). Similarly, GABA-mediated homoexchange was not inhibited by L-*trans*-2,3-PDC (300 μM). Additional experiments demonstrated that the efflux of D-[^{3}H]aspartate or [^{3}H]GABA from the synaptosomes was not altered by the presence of NMDA (100 μM), KA (100 μM), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, 100 μM), or *trans*-aminocyclopentane-1,3-dicarboxylate (trans-ACPD, 100 μM), ruling out the potential contribution of EAA receptors to the exchange process (data not shown).

Substrate and Nonsubstrate Activity of Analogs in Oocytes Expressing EAAT1–3. The actions of the analogs were also examined in *X. laevis* oocytes expressing the cloned human glutamate transporters EAAT1, EAAT2, and EAAT3. Because of the electrogenic nature of the translocation of L-glutamate and associated counter-ions by the EAATs, substrates could be readily differentiated from nonsubstrates based upon the ability to produce an electrical current (Arriza et al., 1994). When 2,4-MPDC was applied to voltage-clamped oocytes expressing EAAT2, this analog induced currents in a dose-dependent manner (Fig. 4A) that yielded a K_m value of 45 μM. This analog proved to be an excellent substrate of EAAT2, as it exhibited an I_{max} value 15% greater than that of L-glutamate itself (Table 2; Esslinger et al., 1998). Although 2,4-MPDC also proved to be a substrate of both EAAT1 and EAAT3, in these instances it exhibited K_m values approximately 2-fold more than those observed at EAAT2 and I_{max} values of only about half those of L-glutamate (Table 2).

In contrast to the actions of 2,4-MPDC, application of 30 μM L-*trans*-2,3-PDC to oocytes expressing EAAT2 did not induce a current (Fig. 4B). However, when coapplied with 30 μM L-glutamate, this analog significantly reduced the substrate-induced currents normally observed with L-glutamate alone. Schild analysis of this inhibition was consistent with a competitive mechanism and yielded a K_d of 16 μM (Fig. 4C). Furthermore, L-*trans*-2,3-PDC did not induce any currents in oocytes expressing EAAT1 or EAAT3 (K_d>300 μM), and was ineffective at inhibiting glutamate-induced currents at these subtypes (data not shown).

Discussion

Much of our understanding of the contribution of sodium-dependent, high-affinity glutamate transporters to EAA physiology and pathology has emerged as a consequence of the ability to pharmacologically manipulate transporter activity. The majority of studies characterizing glutamate transport inhibitors, however, have often neglected to address whether or not the selective blockers are also transported (i.e., whether they also act as substrates for the transporter). Although this limitation has been overcome by electrophysiologically recording the substrate-induced currents in voltage-clamped oocytes expressing cloned transporters (Arriza et al., 1994), it remains a significant obstacle in more complex physiological preparations obtained directly from CNS tissues (e.g., synaptosomes, tissue slices, primary cell culture). In the present study, we demonstrate that transporter-mediated heteroexchange can be readily used in a classical synaptosomal preparation to distinguish transportable from nontransportable inhibitors. The validity of this approach rests on the pretense that the observed synaptosomal efflux of D-[^{3}H]aspartate that occurs as a result of an externally applied compound represents transporter-mediated exchange of internal for external substrate. That this

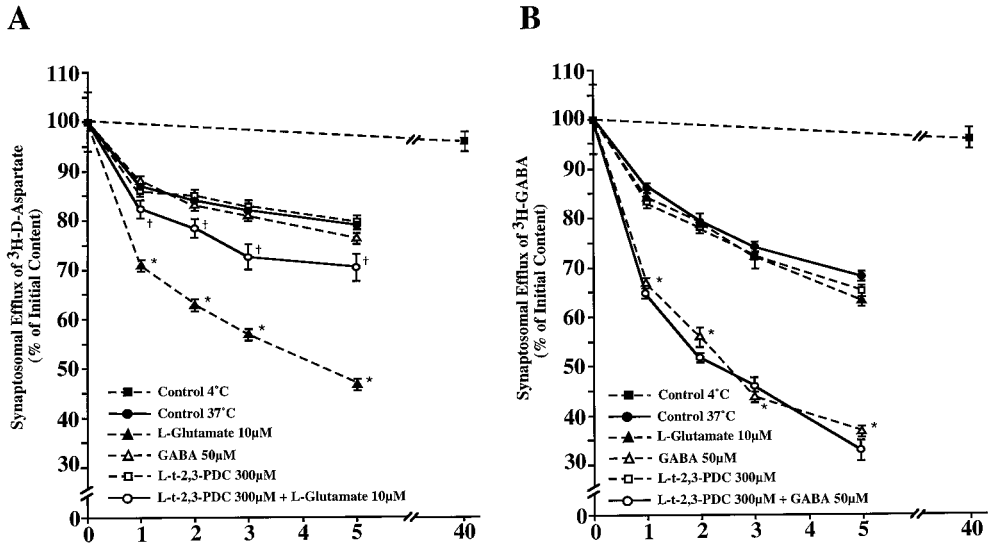

Fig. 3. The time course for the efflux of either (A) D-[^{3}H]aspartate or (B) [^{3}H]-GABA is reported as a percentage of the initial synaptosomal content (1552 $\pm$ 65 pmol D-[^{3}H]aspartate/mg of protein or 3887 $\pm$ 263 pmol [^{3}H]GABA/mg of protein). Although the efflux of ^{3}H-substrate was not detected from synaptosomes maintained at 4°C, the inclusion of L-glutamate or GABA at 37°C caused an efflux of over 50% of synaptosomal D-[^{3}H]aspartate and [^{3}H]GABA, respectively. In contrast, GABA did not stimulate the efflux of D-[^{3}H]aspartate nor did L-glutamate potentiate the release of [^{3}H]GABA. Interestingly, L-*trans*-2,3-PDC did not participate in heteroexchange on its own, but it did significantly attenuate the efflux of D-[^{3}H]aspartate caused by the inclusion of L-glutamate. L-*trans*-2,3-PDC had no effect on the efflux of [^{3}H]GABA. Values are reported as mean $\pm$ S.E. from at least four determinations conducted in duplicate. Statistical comparisons were performed using the Alternate Welch *t* test (InStat): *, $p < .0001$ versus control efflux; †, $p < .01$ versus L-glutamate-induced efflux.

was indeed occurring in the present experiments is supported by the demonstration that:1) efflux was stimulated in a substrate-specific manner (e.g., L-glutamate and L-aspartate, but not GABA, are active); 2) efflux was unaffected by the EAA receptor agonists *N*-methyl-D-aspartate, KA, AMPA, or *trans*-ACPD; and 3) substrate-induced increases in efflux could be attenuated by analogs identified electrophysiologically and biochemically as nontransportable inhibitors (e.g., DHK, L-*trans*-2,3-PDC) (Johnston et al., 1979; Arriza et al., 1994). Thus, compounds could be selectively tested for two distinct steps in the synaptosomal uptake process: competitive inhibition of uptake as an indicator of binding and the stimulation of heteroexchange as a measure of substrate activity.

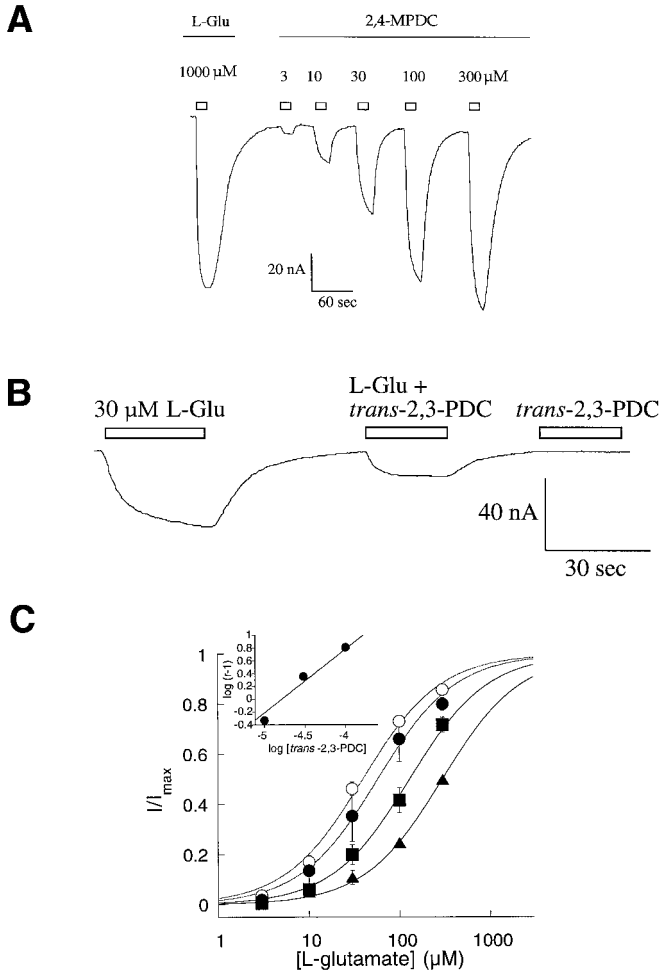

Fig. 4. Actions of transported and nontransported analogs at EAAT2. A, currents induced by application of glutamate and 2,4-MPDC (duration indicated by bar above trace) in representative *X. laevis* oocyte expressing EAAT2. 2,4-MPDC induced a dose-dependent and saturable current with a K_m of 45 μM (membrane potential = −25 mV). B, coapplication of L-*trans*-2,3-PDC (30 μM) reduced glutamate-induced current without inducing current on its own. C, Schild analysis of the effect of L-*trans*-2,3-PDC on glutamate currents revealed competitive antagonism, K_d = 16 μM. ○, control; ●, 10 μM L-*trans*-2,3-PDC; ■, 30 μM L-*trans*-2,3-PDC; ▲, 100 μM L-*trans*-2,3-PDC.

Comparisons of these two properties within the context of our library of conformationally constrained analogs suggest that the ability of a compound to bind to the transporter does not necessarily dictate its activity as a substrate. This is readily exemplified by L-glutamate, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC, each of which competitively inhibited uptake with comparable K_i values ($\approx$ 5–7 μM). Thus, while these compounds seem equal in their ability to bind to the transporter, 2,4-MPDC and L-*anti-endo*-3,4-MPDC were translocated only about 80% and 35% as effectively as L-glutamate, respectively. Such differences in substrate activity were not limited to just those compounds exhibiting K_i values similar to L-glutamate. For example, L-aspartate, L-*trans*-2,4-PDC, and DL-β-THA each inhibited the uptake of D-[^{3}H]aspartate more potently than L-glutamate (e.g., K_i values 1.5–2 μM), yet varied greatly in their ability to stimulate exchange (e.g., 80, 60, and 47% the activity of L-glutamate, respectively). Even more surprising than the substrate activity of these potent inhibitors was the finding that the moderate-to-weak inhibitors ($K_i \approx 30–70$ μM) also included both substrates (*cis*-ACBD, L-*cis*-2,4-PDC, L-*trans*-2,3-ADC) and nonsubstrate inhibitors (e.g., *cis*-5-Me-L-*trans*-2,3-PDC, L-*trans*-2,3-PDC, DHK, and KA). Thus, although a compound such as L-*cis*-2,4-PDC (K_i = 66 μM) may be considered inferior in its ability to initially bind to the substrate site of the transporter, once it occupies this site, it can be translocated as effectively as substrates such as L-aspartate. These findings are also consistent with previous demonstrations that L-*trans*-2,4-PDC and *cis*-ACBD can stimulate the efflux of D-[^{3}H]aspartate from cultured cerebellar granule cells (Griffiths et al., 1994). Likewise, L-*trans*-2,4-PDC has been shown to exchange with L-[^{3}H]glutamate in reconstituted liposomes containing glutamate transporters (Volterra et al., 1996).

Although numerous chemical properties may influence whether or not a compound that binds to the transport protein is also translocated by the uptake system, the present library of analogs demonstrates that conformational restriction does not inherently differentiate transportable from nontransportable inhibitors. For example, the conformational

TABLE 2

Correlation between synaptosomal and EAAT pharmacology

Analogs were evaluated as inhibitors and substrates in oocytes expressing EAAT1, EAAT2, and EAAT3. In the instances where compounds exhibited no substrate activity (*), K_i values were calculated by Schild analysis. In the heteroexchange studies, analogs were tested at concentrations $\approx 10 \times$ greater than the K_i values for inhibition of D-aspartate uptake. Values indicating substrate activity are reported as a percentage of the activity of L-glutamate at 37°C.

Compound	Synaptosomes		EAAT 1		EAAT 2		EAAT 3	
	K_i	efflux	K_m	I_{max}	K_m or K_i*	I_{max}	K_m	I_{max}
	µM	*% of Glu*	*µM*	*% of Glu*	*µM*	*% of Glu*	*µM*	*% of Glu*
L-Glutamate	4.9 ± 1	100	20 ± 3	100	18 ± 3	100	28 ± 6	100
DHK	28 ± 2	3.0 ± 1.5	n.d.	n.d.	9*	0	n.d.	n.d.
L-*trans*-2,3-PDC	33 ± 6	5.0 ± 1.5	n.d.	n.d.	12*	0	n.d.	n.d.
2,4-MPDC	6.8 ± 3	82 ± 9	87 ± 7	40 ± 4	45 ± 3	115 ± 3	85 ± 10	54 ± 2

n.d., not detected.

rigidity produced through the incorporation of distinct pyrrolidine-containing, bicyclic ring systems in 2,4-MPDC and L-*anti-endo*-3,4-MPDC resulted in a similar positioning of their functional groups and comparable K_i values as competitive inhibitors, yet 2,4-MPDC proved to be a very good substrate, whereas L-*anti-endo*-3,4-MPDC did not. Indeed, in a previous study with *X. laevis* oocytes, L-*anti-endo*-3,4-MPDC was found to be a nontransportable inhibitor ($K_i = 1.6\ \mu M$) at EAAT2 (Esslinger et al., 1998). Advantageously, the identification of representative transportable and nontransportable inhibitors that are conformationally rigid allows pharmacophore modeling to be extended beyond the process of binding and toward an initial delineation of the underlying chemical properties that influence substrate activity. To accomplish this, energy-minimized conformations of L-glutamate and representative substrates (2,4-MPDC, L-*trans*-2,4-PDC, and *cis*-ACBD) and nontransportable inhibitors (L-*trans*-2,3-PDC and L-*anti-endo*-3,4-MPDC) were first identified and then systematically overlaid using a Silicon Graphics workstation and SYBYL modeling software (Tripos, St. Louis, MO). The spatial positioning of the two negatively charged carboxylate functionalities (Fig. 5, red) and the positively charged ammonium functionality (Fig. 5, blue) of each analog were compared in a three-point, best-fit analysis to identify conformers exhibiting the greatest degree of overlap. This approach is identical with that used previously to characterize the glutamate transporter binding site pharmacophore (Bridges et al., 1993; Chamberlin et al., 1998; Esslinger et al., 1998). Because all substrates must first bind, the same conformer of glutamate was used as a starting point in both models. As shown in Fig. 5B, the substrates [L-*trans*-2,4-PDC (Fig. 5B, red), 2,4-MPDC (Fig. 5B, yellow), and *cis*-ACBD (Fig. 5B, white)] exhibited a high degree of overlap (e.g., root-mean-square deviation of 0.42 ± 0.17) with the minimized L-glutamate (Fig. 5B, green) conformation of the currently proposed pharmacophore. Similarly, the inhibitors identified as nonsubstrates [L-*trans*-2,3-PDC (Fig. 5B, orange) and L-*anti-endo*-3,4-MPDC (Fig. 5B, light blue)] also overlaid well with this conformation of L–glutamate (Fig. 5B, green), as shown in Fig. 5C. When the overlays of both substrates and nonsubstrate inhibitors were compared (Figs. 5A and 5D), it was noticed that although the functional group positioning was very similar (e.g., root-mean-square deviation of 0.32 ± 0.12), regions of excess steric volume associated with the carbon backbones of the nonsubstrates could be identified that were distinct relative to the space occupied by the backbones of the substrates. These regions become more obvious when molecular volumes of the substrates (Fig 5D, yellow framework) are overlaid with those of the nonsubstrates (Fig 5D, red framework). We suggest that because of the rigidity of the compounds, it is likely that steric excess in these specific positions represents a determining (or at least predictive) factor in whether or not the analogs can be translocated. Thus, although the similar positioning of the charged functionalities allows binding to occur, the excess volume occupied by the backbones of those analogs identified as nontransportable inhibitors may sterically interact in an unfavorable manner with specific domains on the transporter protein that participate in translocating substrates once they are bound.

The electrophysiological characterization of transport into oocytes expressing human EAAT1, EAAT2, or EAAT3 provided an alternative approach to corroborate our conclusions regarding substrate activity as determined by heteroexchange as well as a strategy to compare the pharmacological profile of synaptosomal uptake with that of the individual transporter subtypes. Using a select series of compounds that included both transportable and nontransportable inhibitors, we found a good correspondence between the ability of an analog to stimulate a synaptosomal efflux of D-[^{3}H]aspartate and its ability to induce substrate-mediated currents in the voltage clamped oocytes, particularly those expressing EAAT2. Further evidence that those compounds identified as substrates are actually translocated is suggested by the fact that the external application of the analogs produced inward currents at the reversal potential for Cl^- (Fig. 4). These results are consistent with a current that is associated with stoichiometrically coupled transport and not caused by a blockade of the uncoupled chloride conductance that follows the binding of nontransported analogs (Wadiche and Kavanaugh, 1998). Confirmation of this interpretation, however, will require direct measurement of radiolabeled flux.

At one extreme, 2,4-MPDC exhibited an I_{max} in EAAT2-expressing oocytes greater than that of L-glutamate itself and proved to be one of the most effective analogs in the synaptosomal exchange assays. At the other extreme, L-*trans*-2,3-PDC (and DHK) was found to be inactive at either producing currents in these same oocytes or stimulating the efflux of D-[^{3}H]aspartate from the synaptosomes, yet was capable of competitively blocking the normal glutamate-mediated response in both systems. In the instance of the analogs that were identified as partial substrates in the exchange assays (e.g., L-*trans*-2,4-PDC and DL-β-THA), previous studies have demonstrated that these same compounds produce intermediate I_{max} values in oocytes expressing EAAT1, EAAT2, and EAAT3 (Arriza et al., 1994). It is 2,4-MPDC, L-*trans*-2,3-PDC, and DHK, however, that provided the most compelling evidence linking synaptosomal pharmacology with EAAT2, particularly within the context of substrate activity. Although comparisons of relative I_{max} values and efflux rates are admittedly qualitative in nature, L-*trans*-2,3-PDC and DHK were essentially inactive at EAAT1 and EAAT3, yet effective nontransportable inhibitors of both EAAT2 and the synaptosomal system. Another nontransportable inhibitor of EAAT2, L-*anti-endo*-3,4-MPDC (Esslinger et al., 1998), produced an efflux rate that was only a third of that produced by L-glutamate. 2,4-MPDC, on the other hand, proved to be an excellent substrate of both EAAT2 and the synaptosomes, yet produced I_{max} values at EAAT1 and EAAT3 that were only about half those of L-glutamate. When similar attempts were made to compare the kinetic constants in the two systems, some difficulties arose because the recordings of the substrate-induced currents in the EAATs typically yielded K_m values, whereas the synaptosomal inhibitory assays resulted in K_i constants. This complication was avoided, however, in the instance of the nontransportable inhibitors, as both approaches yield K_i values. In the present study, we found that the K_i values for L-*trans*-2,3-PDC and DHK were very similar to one another within each system, but about 3-fold less in the oocytes than in the synaptosomes. Such a relationship is consistent with recent studies demonstrating that DHK, KA, and DL-*threo*-β-benzyloxyaspartate, all of which are nontransportable inhibitors at EAAT2, also exhibited lower K_i values when characterized in oocytes rather than mamma-

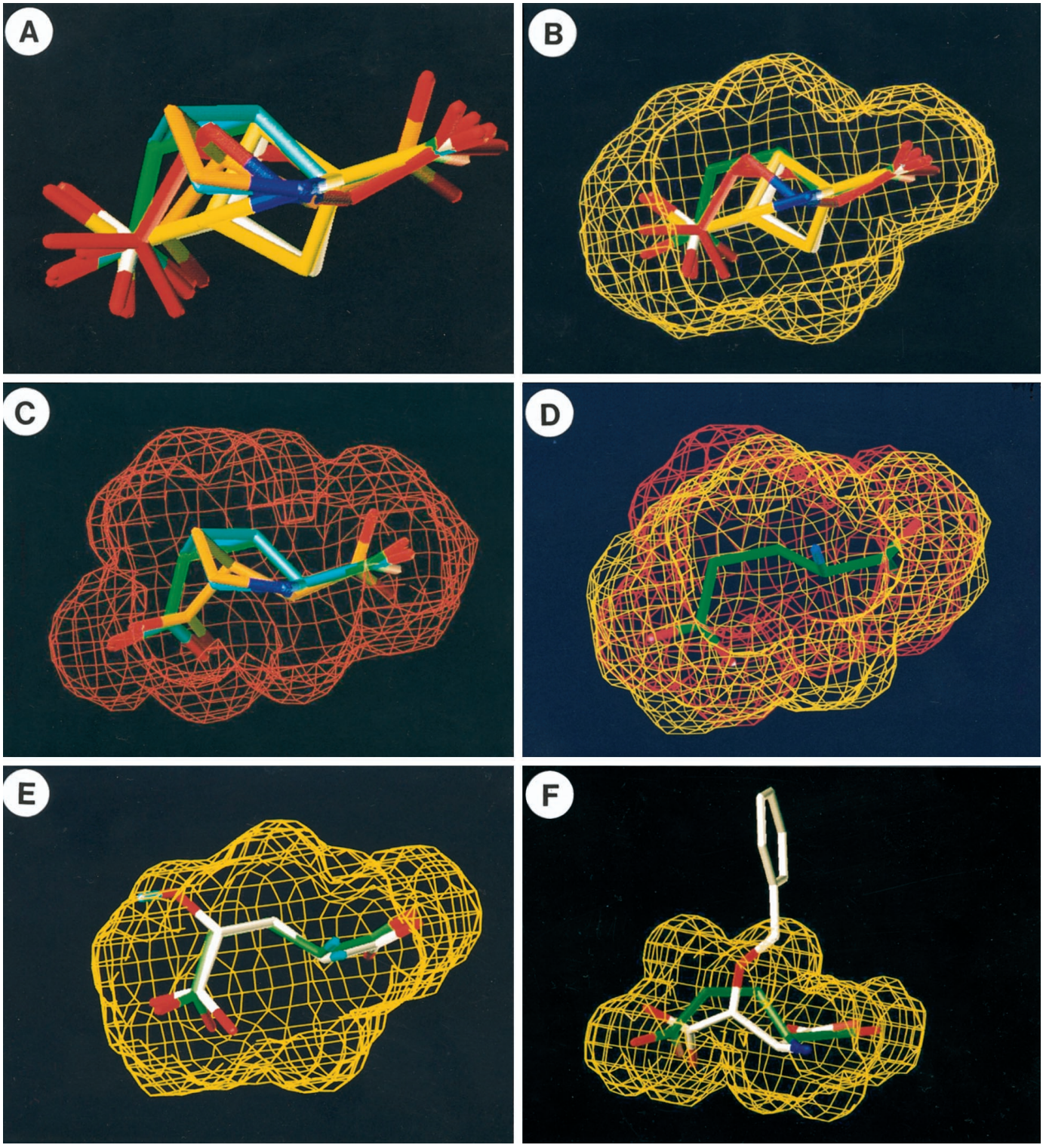

Fig. 5. Molecular modeling overlays using a three-point fit of the amino group (blue), α-carboxylate carbon and distal carboxylate carbon (red oxygens) of inhibitors (A): L-glutamate (green), L-*trans*-2,4-PDC (red), *cis*-ACBD (white); 2,4-MPDC (yellow); L-*anti-endo*-3,4-MPDC (blue); and L-*trans*-2,3-PDC (orange); (B) L-glutamate (green), and substrates: L-*trans*-2,4-PDC (red), *cis*-ACBD (white), and 2,4-MPDC (yellow); (C) L-glutamate (green), and nontransportable inhibitors: L-*anti-endo*-3,4-MPDC (blue), and L-*trans*-2,3-PDC (orange); (D) glutamate (green) and the volume overlays of the substrates (yellow framework) and nontransportable inhibitors (red framework); (E) glutamate (green) and L-*threo*-4-hydroxyglutamate (white) within the substrate volume; and (F) glutamate (green) and DL-*threo*-β-benzyloxyaspartate (white) within the substrate volume. This difference between the substrate volumes (yellow framework) and the nontransportable inhibitor volumes (red framework) are thought to represent regions of excess steric bulk that interact with the transport protein in such a manner as to reduce the substrate activity of these compounds. Conformations depicted were minimized using SYBYL molecular modeling software (Tripos Inc.) as described in the text. Hydrogens were omitted for clarity.

lian cells expressing the transporters (Arriza et al., 1994; Shimamoto et al., 1998).

The conclusion that EAAT2 corresponds most closely with the rat forebrain synaptosomal system is quite interesting in view of the general conclusion that EAAT2 (rat homolog GLT-1), is thought to be chiefly expressed in astrocytes (for review, see Gegelashvili and Schousboe, 1998). The findings suggest, then, that either the synaptosomal preparation contains glial fragments possessing functional transporters or that a separate yet unidentified glutamate transporter subtype exists in neurons that has a pharmacology similar to that of EAAT2. Although the latter can not be ruled out, numerous reports indicate both the heterogeneous nature of synaptosomes (Henn et al., 1976; Nakamura et al., 1993) and that the predominant transport activity in similar forebrain preparations is "EAAT2-like" in its pharmacology (Dowd et al., 1996; Robinson and Dowd, 1997; Gegelashvili and Schousboe, 1998). Significantly, it has also been demonstrated that synaptosomes prepared from mice deficient in GLT-1 exhibited a marked loss in the ability to transport L-glutamate (Tanaka et al., 1997). Regardless of the exact subtype(s) present in the synaptosomes, our data clearly indicates that within the context of the known transporter subtypes, the proposed pharmacophores (as well as the synaptosomal assay system) should be considered working models for the EAAT2 subtype. In this regard, preliminary modeling studies indicate that inhibitors of EAAT2 identified more recently (e.g., DL-*threo*-β-benzyloxyaspartate, L-*threo*-4-hydroxyglutamate, (±)-*threo*-3-methylglutamate, 2*S*,4*R*-4-methylglutamate, and (±)-4-methyleneglutamate; Vandenberg et al., 1997; Shimamoto et al., 1998) also exhibit significant overlap with respect to functional group positionings. Interestingly, the inhibitor identified as an effective EAAT2 substrate, L-*threo*-4-hydroxyglutamate, fits within the proposed substrate pharmacophore (Fig. 5E), whereas the benzyl group on the side chain of the nontranportable inhibitor DL-*threo*-β-benzyloxyaspartate clearly does not (Fig. 5F). As these models are refined for each transporter subtype, they should prove valuable in the design of more selective substrates and nontransportable inhibitors, as well as identify important functional domains that must be incorporated into evolving structural models of the transporters.

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