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Nontransportable Inhibitors Attenuate Reversal of Glutamate Uptake in Synaptosomes Following a Metabolic Insult

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ABSTRACT

Na^+-dependent, high-affinity glutamate transporters in the central nervous system are generally credited with regulating extracellular levels of L-glutamate and maintaining concentrations below those that would induce excitotoxic injury. Under pathological conditions, however, it has been suggested that these same transporters may contribute to excitotoxic injury by serving as sites of efflux for cellular L-glutamate. In this study, we examine the efflux of [^{3}H]D-aspartate from synaptosomes in response to both alternative substrates (i.e., heteroexchange), such as L-glutamate, and a metabolic insult (5 mM potassium cyanide and 1 mM iodoacetate). Exposure of synaptosomes containing [^{3}H]D-aspartate to either L-glutamate or metabolic inhibitors increased the efflux of the radiolabeled substrate to over 200% of control values. Two previously identified competitive transport inhibitors (L-*trans*-2,3-pyrrolidine dicarboxylate and dihydrokainate) failed to stimulate [^{3}H]D-aspartate efflux but did inhibit glutamate-mediated heteroexchange, consistent with the action of nontransportable inhibitors. These compounds also attenuated the efflux of [^{3}H]D-aspartate from synaptosomes exposed to the metabolic inhibitors. These results add further strength to the model of central nervous system injury-induced efflux of L-glutamate through its high-affinity transporters and identify a novel strategy to attenuate this process.

Glutamate is recognized as the primary excitatory amino acid (EAA) neurotransmitter in the mammalian central nervous system, participating in fast synaptic communication as well as the higher-order signal processing required in development, plasticity, learning, and memory (for review see Cotman et al., 1995). In contrast to these physiological roles, the accumulation of excessive levels of extracellular L-glutamate has been shown to be neurotoxic as a consequence of the overactivation of ionotropic EAA receptors (Choi, 1994). Accumulating evidence indicates that this process, referred to as excitotoxicity, is an underlying pathological mechanism in both acute (e.g., trauma and ischemia) and chronic (e.g., Huntington's disease, Alzheimer's disease, and amyotrophic lateral sclerosis) central nervous system (CNS) disorders (Choi, 1994; Rothman and Olney, 1995). High-affinity glutamate uptake systems are believed to play a central role in mediating the balance between the physiological and pathological actions of this excitatory transmitter. The rapid clearance of L-glutamate from the extracellular space into either neurons or glia has been postulated to contribute to signal termination, recycling of the neurotransmitter, and the maintenance of subexcitotoxic levels of L-glutamate (Takahashi et al., 1997). The most prominent of these carriers in the CNS, several subtypes of which have now been cloned (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Arriza et al., 1994), are sodium dependent, and use ionic gradients generated by Na^+-K^+ ATPases to accumulate high intracellular concentrations of L-glutamate. Consistent with a regulatory or protective role of glutamate transporters, reduction in function, as modeled with competitive inhibitors, has been found to increase both the extracellular levels of L-glutamate and the likelihood of excitotoxic injury (Robinson et al., 1993; Rothstein et al., 1993; Amin and Pearce, 1997; Obrenovitch et al., 1997).

Ironically, under some pathological conditions these same high-affinity, sodium-dependent uptake systems may actually participate in the excitotoxic process by acting as sites of efflux of L-glutamate from intracellular compartments (Takahashi et al., 1997). Numerous studies have suggested that metabolic insults that compromise cellular energy levels can lead to the reversed action of the transporter and the movement of L-glutamate down its concentration gradient into the extracellular space (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Gemba et al., 1994; Szatkowski and

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ABBREVIATIONS: ACPD, aminocyclopentane dicarboxylate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NMDA, *N*-methyl-D-aspartate; ASP, aspartate; DHK, dihydrokainate; EAA, excitatory amino acid; IOA, iodoacetate; PDC, pyrrolidine dicarboxylate; THA, *threo*-hydroxy-aspartate.

Attwell, 1994; Longuemare and Swanson, 1995). This pathway, possibly in combination with alternative routes of L-glutamate efflux (Kimelberg et al., 1990), could contribute to the rise in extracellular glutamate levels observed in models of anoxia and ischemia (Benveniste et al., 1984; Roettger and Lipton, 1996). In the present study we examine the efflux of [^{3}H]D-aspartate ([^{3}H]D-ASP), a selective substrate of the high-affinity, sodium-dependent glutamate carriers, from synaptosomes in response to both alternative substrates (i.e., heteroexchange) and a metabolic insult (i.e., potassium cyanide (KCN) and iodoacetate (I0A)). A subtype of transport inhibitor is also identified that competitively binds to the glutamate carrier but does not appear to be translocated into synaptosomes (i.e., nontransportable inhibitors). We demonstrate that the heteroexchange-mediated and metabolic insult-mediated efflux of [^{3}H]D-ASP from the synaptosomes exhibit similar time courses and that both processes can be attenuated with nontransportable uptake inhibitors. These results add further strength to the model of injury-induced efflux through the glutamate carriers and identify a novel strategy to attenuate this process.

Materials and Methods

Synaptosomes were prepared from rat forebrain essentially by the procedure of Booth and Clark (1978), using a discontinuous Ficoll/sucrose gradient as previously described (Bridges et al., 1994). Isolated 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 $MgSO_4$, and 1 mM $CaCl_2$, pH 7.4) to 0.45 mg protein/ml. Aliquots of this suspension (V_{tot} = 10 ml) were allowed to incubate with either 2.5 μM [^{3}H]D-ASP 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 (1 mg protein/ml) in ice-cold assay buffer, and maintained on ice. Initial content of radiolabeled substrates in the synaptosomes (i.e., time 0) was determined by adding 100 μl of the suspension to 2.9 ml of ice-cold assay buffer and immediately vacuum filtering through glass microfiber filters (Whatman GF/F). After rinsing with an additional 4 ml of ice-cold assay buffer, filters were transferred to vials containing 4.0 ml of scintillation fluid (National Diagnostics, Atlanta, GA) and allowed to stand for 24 h. Radioactivity retained in the synaptosomes was quantified by liquid scintillation counting. Similar measurements were taken throughout each experiment to ensure that the synaptosomal content of either [^{3}H]D-ASP or [^{3}H]GABA did not change during maintenance on ice and that different preparations contained similar levels of each of the radiolabeled substrates. Assays quantifying efflux of [^{3}H]D-ASP or [^{3}H]GABA from the synaptosomes were initiated by adding a 100-μl aliquot of the appropriate suspension to 2.9 ml of assay buffer (37°C) in the presence or absence of the indicated compounds. Assays were terminated 1 to 5 min later by the addition of 4.0 ml of ice-cold assay buffer and the retained radioactivity determined as described above. Efflux rates were normalized to protein content as determined by the Pierce (Rockford, IL) bicinchoninic acid assay (Smith et al., 1985). Statistical analyses were made with GraphPad (San Diego, CA) software using an Alternate Welch *t* test that allowed populations with means of unequal S.D.s to be compared. L-*trans*-2,4-Pyrrolidine dicarboxylate (PDC) and −2,3-PDC were prepared as described previously (Bridges et al., 1991; Humphrey et al., 1994). α-Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), *N*-methyl-D-aspartate (NMDA), and (±)-1-aminocyclopentane-*trans*-1,3-dicarboxylate (ACPD) were obtained from Tocris (Ballwin, MO). All other reagents were obtained from Sigma (St. Louis, MO).

Results and Discussion

Early studies on the transport of L-glutamate demonstrated that the addition of one substrate could stimulate the efflux of a second substrate that had been previously accumulated by the membrane preparation (Kanner and Marva, 1982; Wilson and Pastuszko, 1986). This process, referred to as heteroexchange, has also been observed in cultured cell systems (Griffiths et al., 1994; Volterra et al., 1996). In the present study, we take advantage of transporter-mediated heteroexchange to distinguish substrate from nonsubstrate inhibitors. The glutamate analog D-aspartate was used in these studies because it is an excellent substrate of the high-affinity glutamate carrier, is not metabolized, and is not a substrate of the synaptic vesicular uptake system (Tabb and Ueda, 1991). Initial assays examined the specificity of [^{3}H]D-ASP heteroexchange by comparing its efflux with that of [^{3}H]GABA. The results of these experiments are summarized in Table 1. Values are reported as a percentage of the efflux observed over a 2-min time period at 37°C in the absence of added compounds (114 ± 4 and 354 ± 30 pmol/min/mg protein for [^{3}H]D-ASP and [^{3}H]GABA, respectively). Importantly, the amount of [^{3}H]D-ASP or [^{3}H]GABA initially accumulated within synaptosomes exhibited little variation among experiments (1552 ± 62 and 3887 ± 263 pmol/mg protein for [^{3}H]D-ASP and [^{3}H]GABA, respectively), and the efflux of either radiolabel was negligible during the course of a single experiment (≤40 min), when synaptosomes were maintained at 4°C (Fig. 1). When the synaptosomes containing [^{3}H]D-ASP were diluted into buffer containing L-glutamate (10 μM), the rate of the efflux was markedly enhanced, consistent with the process of heteroexchange (Table 1). In contrast, the rate of efflux of [^{3}H]D-ASP was not enhanced in

TABLE 1
Differentiation of substrate and nonsubstrate transport inhibitors by heteroexchange

Efflux of radiolabeled substrate from synaptosomes containing either [^{3}H]D-ASP or [^{3}H]GABA was determined as described in *Materials and Methods* and is expressed as percentage of control (i.e., efflux over a 2-min interval at 37°C in the absence of uptake inhibitors). Initial content of synaptosomes was 1552 ± 62 pmol [^{3}H]D-ASP/mg protein or 3887 ± 263 pmol [^{3}H]GABA/mg protein. Control efflux rates were 114 ± 4 pmol/min/mg protein for [^{3}H]D-ASP and 354 ± 30 pmol/min/mg for [^{3}H]GABA. Values are reported as mean ± S.E.M., n = 4-57 duplicate determinations. Statistical comparisons were made using an Alternate Welch *t* test (InStat).

Experiment	[^{3}H]D-Aspartate Efflux	[^{3}H]GABA Efflux
	% of control	*% of control*
Control	100 ± 4	100 ± 8
L-Glutamate, 10 μM	233 ± 10[a]	100 ± 6
GABA, 50 μM	102 ± 11	252 ± 18[c]
Kainate, 100 μM	81 ± 6	104 ± 8
AMPA, 100 μM	97 ± 6	99 ± 7
NMDA, 100 μM	91 ± 3	99 ± 7
trans-ACPD, 100 μM	100 ± 10	99 ± 7
β-D,L-THA, 20 μM	167 ± 8[a]	102 ± 5
L-*trans*-2,4-PDC, 15 μM	186 ± 17[a]	96 ± 4
L-*trans*-2,3-PDC, 300 μM	96 ± 5	95 ± 6
DHK, 300 μM	94 ± 6	101 ± 2
L-Glutamate, 10 μM and L-*trans*-2,3-PDC, 300 μM	129 ± 7[b]	N.D.
L-Glutamate, 10 μM and DHK, 300 μM	129 ± 8[b]	N.D.
GABA, 50 μM and L-*trans*-2,3-PDC, 300 μM	N.D.	248 ± 28[c]
GABA, 50 μM and DHK, 300 μM	N.D.	252 ± 20[c]

[a] $p < .0005$ versus [^{3}H]D-ASP control; [b] $p < .0001$ versus glutamate-mediated [^{3}H]D-ASP efflux; [c] $p < .001$ versus [^{3}H]GABA control. N.D., not determined.

the presence of GABA (50 μm). Analogously, the efflux of [^{3}H]GABA was stimulated by the inclusion of GABA (homoexchange), but not by L-glutamate (Table 1). Failure of the EAA agonists kainate, AMPA, NMDA, or *trans*-ACPD to stimulate efflux of either [^{3}H]D-ASP or [^{3}H]GABA indicates that the observed effects were not a consequence of EAA receptor activation. To assure comparable levels of occupancy of the substrate binding sites, competitive transport inhibitors were included in the assays at concentrations approximating 10-fold the K_i value for inhibition of synaptosomal [^{3}H]D-ASP uptake [i.e., β-DL-*threo*-hydroxy-aspartate (THA), 2.0 $\pm$ 1 μM; L-*trans*-2,4-PDC, 1.5 $\pm$ 0.5 μM, L-*trans*-2,3-PDC, 33 $\pm$ 6 μM; and dihydrokainate (DHK), 28 $\pm$ 2 μM; data not shown]. Of these inhibitors, β-DL-THA and L-*trans*-2,4-PDC significantly stimulated the efflux of [^{3}H]D-ASP (but not [^{3}H]GABA). In contrast, the efflux of [^{3}H]D-ASP in the presence of either L-*trans*-2,3-PDC or DHK could not be distinguished from control values. Furthermore, the inclusion of either L-*trans*-2,3-PDC or DHK at a concentration of 300 μM attenuated the increase in efflux of [^{3}H]D-ASP that was produced by 10 μM L-glutamate. Even though L-*trans*-2,3-PDC and DHK failed to stimulate heteroexchange, both of these previously identified uptake inhibitors effectively blocked glutamate, but not GABA-mediated exchange. These results demonstrate that although all of the inhibitors bind to the glutamate transporter, a distinction can be made between β-DL-THA and L-*trans*-2,4-PDC as substrates for the transporter and L-*trans*-2,3-PDC and DHK as nontransportable inhibitors. Such a conclusion is consistent with previous studies identifying β-DL-THA and L-*trans*-2,4-PDC as transporter substrates based upon an ability to participate in the process of heteroexchange in cultured astrocytes (Griffiths et al., 1994), hippocampal slices (Roettger and Lipton, 1996), or reconstituted liposomes (Volterra et al., 1996). Furthermore, both β-DL-THA and L-*trans*-2,4-PDC have also been shown to produce susbtrate-mediated currents in oocytes expressing the human transporter clones EAAT1, EAAT2, and EAAT3 (Arriza et al., 1994). In contrast, DHK (Arriza et al., 1994) and L-*trans*-2,3-PDC (Bridges et al., 1996) were found to selectively block glutamate-induced currents at EAAT2, yet did not induce currents when applied alone, as would be expected of nontransportable inhibitors.

A stimulation of the efflux of preaccumulated [^{3}H]D-ASP was also observed when synaptosomes were exposed to a chemical insult consisting of KCN (5 mM) and iodoacetate (IOA, 1 mM) (Table 2). The combination of an electron transport chain blocker (KCN) and a glycolytic inhibitor (IOA) has been employed in numerous studies as a metabolic insult and as a simplified chemical model of ischemia/anoxia because of its ability to inhibit respiration, deplete ATP levels, and erode membrane potentials (Kauppinen et al., 1988; Reiner et al., 1990; Zeevalk and Nicklas, 1991; Longuemare and Swanson, 1995). As illustrated in Fig. 1, the efflux of [^{3}H]D-ASP produced by KCN and IOA exhibited a time course very similar to that of glutamate-mediated heteroexchange. Over a 5-min period the synaptosomal content of [^{3}H]D-ASP was reduced to about 50% of its original level. Interestingly, an analogous increase in efflux was not observed when synaptosomes containing [^{3}H]GABA were exposed to these same metabolic inhibitors (Fig. 1). Whether this lack of response was attributable to: 1) the metabolism or sequestration of GABA in synaptic vesicles (unlikely, given the extent of the homoexchange-mediated efflux); 2) the GABA pool being less sensitive to metabolic insult, as suggested by Hauptman et al. (1984); or 3) as a consequence of the inhibitor-induced efflux of [^{3}H]GABA being masked by a larger basal rate of efflux (Fig. 1), remains to be determined. Although the potential action of KCN and IOA at alternative sites cannot be excluded, the results are consistent with previous demonstrations that chemical inhibition of cellular respiration increases extracellular levels of L-glutamate in a variety of

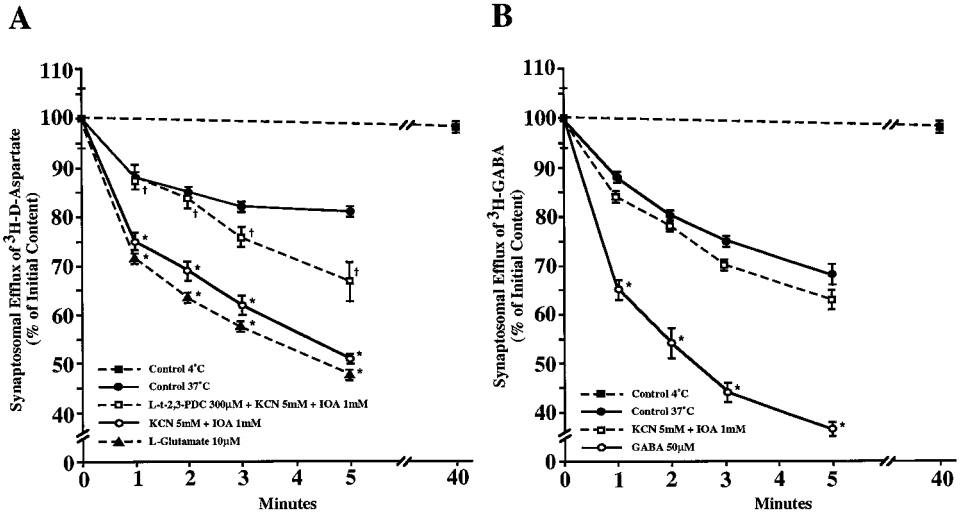

Fig. 1. Synaptosomal efflux of [^{3}H]D-ASP (A) or [^{3}H]GABA (B), reported as a percentage of initial radiolabel content (1552 $\pm$ 65 pmol [^{3}H]D-ASP/mg protein and 3887 $\pm$ 263 pmol [^{3}H]GABA/mg protein), was followed over a period of 5 min in the presence and absence of indicated compounds. Synaptosomes maintained at 4°C did not exhibit a significant loss of [^{3}H]substrate, whereas the inclusion of L-glutamate or GABA at 37°C caused an efflux of over 50% of synaptosomal [^{3}H]D-ASP or [^{3}H]GABA, respectively. A metabolic insult of 5 mM KCN and 1 mM IOA caused an efflux of [^{3}H]D-ASP similar in extent to that caused by 10 μM L-glutamate. In contrast, 5 mM KCN and 1 mM IOA caused only a slight, albeit statistically nonsignificant, increase in the efflux of [^{3}H]GABA. Interestingly, inclusion of the nontransportable uptake inhibitor L-*trans*-2,3-PDC significantly attenuated the efflux of [^{3}H]D-ASP that was caused by the metabolic insult. Values are reported as mean $\pm$ S.E.M. from at least four duplicate determinations. Statistical comparisons were made using an Alternate Welch *t* test (InStat) that does not assume equal SDs: *$p < .0001$ versus control efflux; †$p < .005$ versus KCN- and IOA-induced efflux.

physiological preparations (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Zeevalk and Nicklas, 1991; Madl and Burgesser, 1993; Gemba et al., 1994).

In addition to an ability to block heteroexchange, the nontransportable inhibitors L-*trans*-2,3-PDC and DHK also attenuated the efflux of [^{3}H]D-ASP induced by the metabolic inhibitors, consistent with the involvement of the same transport system in both processes (Table 2 and Fig. 1). In contrast to the ability of analogs to block glutamate-mediated heteroexchange by directly competing with extracellular L-glutamate for carrier binding sites, these results lead to the conclusion that occupation of external binding sites by nontranslocatable analogs reduces the rate at which an internal substrate can egress through this system. Mechanistically, this process is also distinct from the reduction in the metabolic inhibitor-induced efflux of [^{3}H]D-ASP from cultured astrocytes that was produced intracellularly by pre-equilibrating the cells with competitive inhibitors that were also substrates (β-DL-THA and L-*trans*-2,4-PDC; Longuemare and Swanson, 1995). It therefore appears that the binding of a nontransportable analog may essentially trap the carrier binding site on the extracellular face of the plasma membrane. In support of this interpretation, a reduction in the metabolic inhibitor-induced efflux was observed only with the nontransportable blockers and not with substrate inhibitors (e.g., β-DL-THA and L-*trans*-2,4-PDC; Table 2). It is also acknowledged that the attenuation of the [^{3}H]D-ASP efflux by L-*trans*-2,3-PDC was not complete (e.g., about 50% at 5 min; Fig. 1), suggesting that a portion of the insult-induced efflux was also occurring via either an EAAT subtype insensitive to L-*trans*-2,3-PDC (see below) or through a non-EAAT-mediated mechanism (Kimelberg et al., 1990).

Interestingly, both DHK (Pines et al., 1992; Arriza et al., 1994; Vandenberg, 1998), and L-*trans*-2,3-PDC (Bridges et al., 1996) appear to be selective inhibitors of the EAAT2/GLT-1 transporter subtype believed to be localized primarily on glia in the CNS (for review see: Gegelashvili and Schousboe, 1998). This specificity would, in turn, suggest that at least some portion of the observed efflux of [^{3}H]D-ASP originated from glial elements within the synaptosomal preparation (see Henn et al., 1976). The presence of glial transporters in this subcellular fraction is also consistent with the demonstration that synaptosomes prepared from mice deficient in GLT-1 exhibited a marked loss in the ability to transport L-glutamate (Tanaka et al., 1997). Although more selective inhibitors and additional kinetic studies will be needed to quantitatively delineate the specific cellular pools and individual proteins that contribute to excitatory amino acid efflux during metabolic insult, these findings highlight the use of nontransportable uptake inhibitors as a novel strategy to regulate this process.

TABLE 2

Attenuation of KCN- and IOA-induced efflux of [^{3}H]D-aspartate from synaptosomes by nontransportable inhibitors

Efflux of synaptosomal [^{3}H]D-ASP was quantified over a 2-min period at 37°C and is reported as percentage of control (i.e., efflux in the absence of added substrates). Initial content of synaptosomes and control rate of efflux are identical to those reported in Table 1. Efflux of [^{3}H]D-ASP induced by a metabolic insult of 5 mM KCN and 1 mM IOA was significantly attenuated by the nontransportable uptake inhibitors L-*trans*-2,3-PDC and DHK but not by the transportable inhibitors β-D,L-THA and L-*trans*-2,4-PDC. Values are reported as mean ± S.E.M., n = 4-57 duplicate determinations. Statistical comparisons were made using an Alternate Welch t test (InStat).

Experiment	[^{3}H]D-Aspartate Efflux
	% of control
Control	100 ± 4
KCN, 5 mM and IOA, 1 mM	224 ± 18[a]
KCN, 5 mM, IOA, 1 mM and L-*trans*-2,3-PDC, 300 μM	117 ± 12[b]
KCN, 5 mM, IOA, 1 mM and DHK, 300 μM	121 ± 17[b]
KCN, 5 mM, IOA, 1 mM and β-D,L-THA, 20 μM	280 ± 42[a]
KCN, 5 mM, IOA, 1 mM and L-*trans*-2,4-PDC, 15 μM	284 ± 32[a]

[a] $p < .0001$ versus control efflux; [b] $p < .005$ versus KCN and IOA induced efflux.

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