

# Dissociation of [<sup>3</sup>H]L-Glutamate Uptake from L-Glutamate-Induced [<sup>3</sup>H]D-Aspartate release by 3-Hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic Acid and 3-Hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic Acid, Two Conformationally Constrained Aspartate and Glutamate Analogs

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## ABSTRACT

We characterized the interaction of two conformationally constrained aspartate and glutamate analogs, 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic acid (HIP-A) and 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid (HIP-B), with excitatory amino acid transporters (EAATs) in rat brain cortex synaptosomes. HIP-A and HIP-B were potent and noncompetitive inhibitors of [<sup>3</sup>H]L-glutamate uptake, with IC<sub>50</sub> values (17–18 μM) very similar to that of the potent EAAT inhibitor DL-threo-β-benzyloxyaspartic acid (TBOA). The two compounds had little effect in inducing [<sup>3</sup>H]D-aspartate release from superfused synaptosomes but they potently inhibited L-glutamate-induced [<sup>3</sup>H]D-aspartate release, thus behaving as EAAT blockers, not substrates, in a manner similar to those of TBOA and dihydrokainate (DHK). HIP-A and HIP-B, but not TBOA and DHK, unexpectedly inhibited L-glutamate-induced [<sup>3</sup>H]D-aspartate release with IC<sub>50</sub> values (1.2–1.6 μM) 10 times lower than those required to inhibit [<sup>3</sup>H]L-glutamate uptake. There is therefore a

concentration window (1–3 μM) in which the two compounds significantly inhibited L-glutamate-induced release with very little effect on L-glutamate uptake. This selective inhibitory effect required quite long preincubation (>5 min) of synaptosomes with the drugs. At these low concentrations, however, HIP-A and HIP-B had no effect on the EAAT-mediated [<sup>3</sup>H]D-aspartate release induced by altering the ion gradients, indicating that they specifically affect some L-glutamate-triggered process(es)—different from L-glutamate translocation itself—responsible for the induction of reverse transport. These data are inconsistent with the classic model of facilitated exchange-diffusion and provide the first evidence that EAAT-mediated substrate uptake and substrate-induced EAAT-mediated reverse transport are independent. Compounds such as HIP-A and HIP-B could be useful to further clarify the mechanisms underlying these operating modes of transporters.

Glutamate is considered the main excitatory transmitter in the mammalian brain and is involved in various physiological functions, including development and plasticity, learning and memory, cognition, pain, and nociception. Abnormally high extracellular glutamate levels, however, cause excitotoxic neuronal death, which involves activation of ionotropic glutamate receptors and excessive calcium influx in

neurons (Choi et al., 1987) and is implicated in numerous neurodegenerative diseases. Glutamate receptor activation is terminated, and extracellular glutamate levels kept below toxic levels, primarily by reuptake into glial cells and neurons through high-affinity Na<sup>+</sup>-dependent excitatory amino acid transporters (EAATs) (Danbolt, 2001).

EAATs can also contribute to neurotoxic glutamate release. Thus, in conditions in which energy levels fall and the transmembrane gradients of Na<sup>+</sup> collapse (e.g., during ischemia), the electrogenic Na<sup>+</sup>-coupled transporters reverse and

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**ABBREVIATIONS:** EAAT, excitatory amino acid transporter; HIP-A, 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic acid; HIP-B, 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid; AMPA, (S)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid; TBOA, DL-threo-β-benzyloxyaspartic acid; DHK, dihydrokainate; CI, confidence interval; FRR, fractional release rate.

release glutamate into the extracellular space (McMahon and Nicholls, 1991; Levi and Raiteri, 1993). Most of the release in early ischemia probably occurs this way (Seki et al., 1999; Phillis et al., 2000; Rossi et al., 2000), and is also referred to as transporter-mediated release (Levi and Raiteri, 1993). Transporter-mediated release of intracellular neurotransmitter can also be elicited by the extracellular presence of transporter substrates (Koch et al., 1999b), according to the model of "facilitated exchange diffusion" or "transporter-mediated hetero-exchange" (Fischer and Cho, 1979; Levi and Raiteri, 1993).

Blockade of EAATs by nontransportable inhibitors (or blockers) affects glutamate uptake and, when operating, also EAAT-mediated release. EAAT blockers with low or no affinity for ionotropic glutamate receptors could therefore be useful to prevent the glutamate release and neuron death after cerebral ischemia but can produce neurotoxicity in physiological conditions.

In the present study, we characterized the interaction of two conformationally constrained aspartate and glutamate analogs (Fig. 1), 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic acid (HIP-A) and 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid (HIP-B), with the EAATs expressed in rat brain cortex synaptosomes, a GLT1-like subtype (Tanaka et al., 1997; Koch et al., 1999a; Suchak et al., 2003). A previous study (Conti et al., 1999b) found that HIP-A and HIP-B had low affinity for (S)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid (AMPA) and kainic acid receptors and none for N-methyl-D-aspartate receptors. HIP-A and HIP-B were also inactive at metabotropic glutamate receptors as agonists or antagonists (Conti et al., 1999b).

We first evaluated the compounds' potency in inhibiting [<sup>3</sup>H]L-glutamate uptake and then carried out release experiments to define these compounds as "substrates" or nontransportable "blockers" of EAATs, as described previously (Koch et al., 1999b). We evaluated the compounds' ability to induce tritium release from superfused synaptosomes preloaded with [<sup>3</sup>H]D-aspartate, which was used because it is an excellent substrate of EAATs, is not metabolized, and is a poor substrate of vesicular glutamate carrier (Naito and Ueda, 1985; Fykse et al., 1992; Bartlett et al., 1998; Koch et al., 1999a). [<sup>3</sup>H]D-aspartate release from synaptosomes is

selectively induced only by EAAT "substrates", according to the proposed process of EAAT-mediated heteroexchange, and no [<sup>3</sup>H]D-aspartate release can be induced by substrates of other transporters or by selective ligands of ionotropic receptors (Koch et al., 1999b). We therefore used L-glutamate as the prototypical EAAT substrate. Compounds acting as EAAT "blockers" can be identified by evaluating their inhibition of L-glutamate-induced, EAAT-mediated, [<sup>3</sup>H]D-aspartate release. DL-threo-β-benzyloxyaspartic acid (TBOA) was used here as the reference compound because it is a potent nontransportable blocker of EAAT<sub>1-3</sub>, with no significant effects on either the ionotropic or metabotropic glutamate receptors (Shimamoto et al., 1998). Dihydrokainate (DHK) was used as a selective EAAT<sub>2</sub>/GLT1 blocker, with no affinity for EAAT<sub>1</sub>/GLAST and EAAT<sub>3</sub>/EAAC1 transporters (Arriza et al., 1994). Finally, we evaluated the compounds' action in inhibiting [<sup>3</sup>H]D-aspartate release induced in superfused synaptosomes by affecting the ion gradients. With the superfusion apparatus we employed (Raiteri et al., 1974), the neurotransmitter released by the drug is immediately removed and cannot interact with presynaptic receptors or be taken up (Gobbi et al., 1992).

## Materials and Methods

**Reagents.** HIP-A and HIP-B were synthesized as reported previously (Conti et al., 1999a). L-glutamic acid, DL-threo-β-hydroxyaspartic acid, 2-deoxy-D-glucose, and rotenone were obtained from Sigma (St Louis, MO). TBOA was obtained from Tocris (Ellisville, MO). DHK was purchased both from Tocris and from Sigma. [<sup>3</sup>H]L-glutamate and [<sup>3</sup>H]D-aspartate were purchased from Amersham Biosciences (Buckinghamshire, UK).

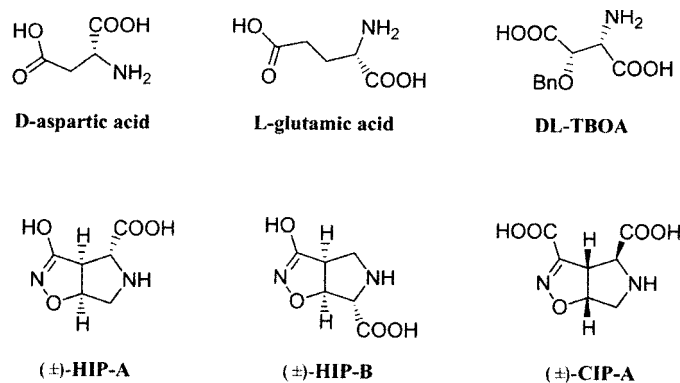
**Animals.** Adult male CRL:CD(SD)BR rats (Charles River, Calco, Italy) were used. They were kept in a controlled environment with a 12-h light/dark cycle and 21°C temperature. Food and water were provided ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n.116 G.U., suppl. 40, 1992 Feb 18) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 1987 Dec 12; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

**Preparation of the Synaptosomal Fraction.** Rats were killed by decapitation and the cortex was rapidly dissected out and homogenized in 40 volumes of ice-chilled 0.32 M sucrose, pH 7.4, in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 1000g for 5 min and the supernatants centrifuged again at 12,000g for 20 min to yield the crude synaptosomal pellet (P2) (Whittaker, 1988).

**Synaptosomal [<sup>3</sup>H]L-Glutamate Uptake.** The P2 pellets were diluted to a concentration of about 3 mg of tissue/ml, wet weight, in assay buffer (Koch et al., 1999b; 10 mM Tris-acetate, 128 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, and 1 mM CaCl<sub>2</sub>, pH 7.4). In some experiments, we used a Ca<sup>2+</sup>-free assay medium or replaced NaCl with LiCl.

Samples of 0.5 ml were preincubated for 7 min (unless otherwise indicated) at 35°C in a water bath with or without the compounds to be tested. Nonspecific uptake was determined in the presence of 300 μM L-glutamate. Uptake was started by adding 10 μM [<sup>3</sup>H]L-glutamate (initial specific activity, 56.0 Ci/mmol; specific activity after isotopic dilution with unlabeled L-glutamate, 0.056 Ci/mmol) or 10 nM [<sup>3</sup>H]D-aspartate (13 Ci/mmol) and was stopped 4 min later by adding 2 ml of ice-chilled assay buffer. In some experiments, we used different concentrations of [<sup>3</sup>H]L-glutamate. L-glutamate uptake was linear over this period of time (Dixon and Hokins, 1998).

Samples were immediately filtered through cellulose mixed ester filters (0.65 μm pore size; Millipore Corporation, Milano, Italy) and



**Fig. 1.** Chemical structures of 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-4-carboxylic acid (HIP-A), 3-hydroxy-4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-6-carboxylic acid (HIP-B) and 4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-3,4-dicarboxylic acid (CIP-A) in comparison with L-glutamic acid, D-aspartic acid, and DL-threo-β-benzyloxyaspartic acid (TBOA).

washed with 2 ml of assay buffer. The radioactivity trapped on the filters was counted in 4 ml of Ultima Gold MV (PerkinElmer Life and Analytical Sciences, Groningen, the Netherlands) in an LKB 1214 Rackbeta liquid scintillation counter with a counting efficiency of about 60%. For each drug, the percentage inhibition of specific uptake were determined for four to six concentrations in at least three different experiments and the inhibition curves were fitted using the "one-site competition" equation built into Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). This analysis gives the  $IC_{50}$  (i.e., the drug concentration inhibiting specific binding by 50%), with its 95% confidence intervals (CI).

**[ $^3H$ ]D-Aspartate Release from Superfused Synaptosomes.** The P2 pellets were resuspended in about 40 volumes of assay buffer (see above) and incubated for 15 min at 37°C with 60 nM [ $^3H$ ]D-aspartate (13.0 Ci/mmol). The suspension was then diluted with fresh buffer and 2.5-ml samples (about 3 mg of initial tissue) were distributed onto cellulose mixed ester filters (0.65- $\mu$ m pore size; Millipore) in a 20-chamber superfusion apparatus held thermostatically at 37°C (Raiteri et al., 1974). The synaptosomes were layered onto the filters by aspiration from the bottom under moderate vacuum. Superfusion was started ( $t = 0$  min) with assay buffer at a rate of 0.5 ml/min; after 24 min equilibration, fractions were collected every 2 min until  $t = 34$  to 42 min. At the end, the filters were put into scintillation vials and counted for radioactivity, as the fractions, in 4 ml of Ultima Gold MV (PerkinElmer Life and Analytical Sciences).

Releasing effects were measured by exposing the superfused synaptosomes to drugs from  $t = 27$  min on. In some experiments, to assess the effects on L-glutamate-induced release, synaptosomes were pre-exposed to the drugs (usually from  $t = 20$  min) before adding 10  $\mu$ M L-glutamate from  $t = 27$  min. In other experiments, [ $^3H$ ]D-aspartate release was induced by conditions that favor reversal of transporter function: 1) inhibition of energy-dependent processes obtained by adding 5  $\mu$ M rotenone and 2 mM 2-deoxy-glucose in glucose-free medium (inhibition of mitochondrial ATP synthesis, glycolysis, and glycogenolysis) (McMahon and Nicholls, 1991; Santos et al., 1996; Koch et al., 1999a); 2) alteration of the ionic gradients, by increasing the  $K^+$  concentration of superfusion buffer from 5 to 35 mM replacing an equimolar  $Na^+$  concentration (Szatkowski et al., 1991; Muzzolini et al., 1997); 3) increase of intracellular  $Na^+$  concentration achieved in the presence of the  $Na^+/H^+$  ionophore monensin, 3  $\mu$ M (Erecinska et al., 1991). As above, drugs were added from  $t = 20$  min, whereas these releasing stimuli were given from  $t = 27$  min on. For these experiments, the superfusion buffer was  $Ca^{2+}$ -free from  $t = 10$  min.

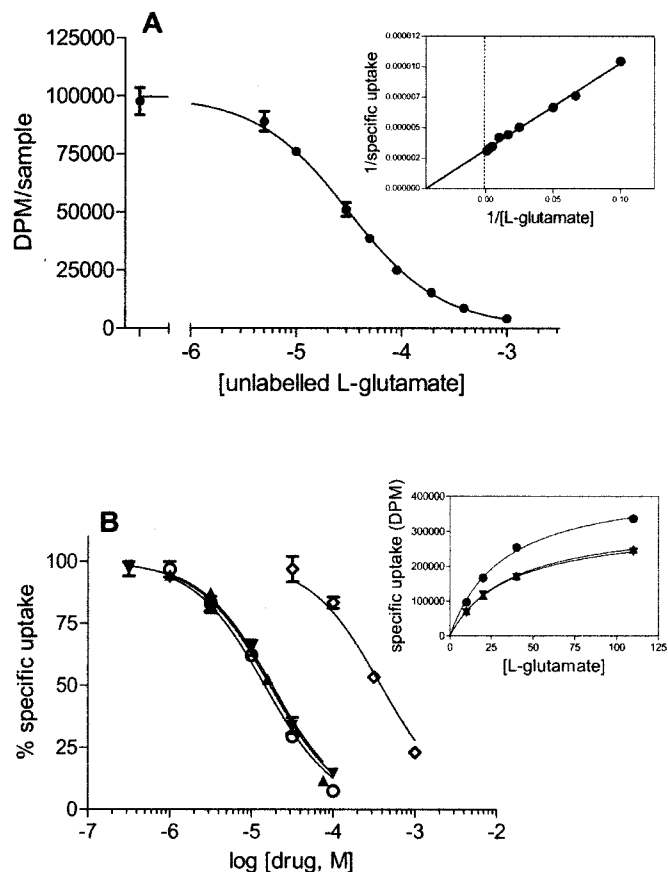
The fractional release rate (FRR) was calculated as 100 times the amount of radioactivity released into each 2-min fraction over the total radioactivity present on the filter at the start of that fraction. The FRRs found before the releasing stimulus (two fractions,  $t = 24$ –26 min and  $t = 26$ –28 min), expressed as a percentage in 2 min, were considered as the basal outflow. The percentage drug-induced release was calculated as the difference between the FRR in the presence of drugs ( $t = 30$ –34 min) and the basal outflow. It should be noted that the effect of the drugs added at  $t = 27$  min was only detectable 1 min later, because the fluid takes about 1.5 min to flow from the filters to the collecting vials.

**Vesicular [ $^3H$ ]L-Glutamate Uptake.** Vesicular [ $^3H$ ]L-glutamate uptake was measured as described previously (Roseth et al., 1998). In brief, freshly prepared vesicles from rat forebrain were diluted to a concentration of about 250 mg of tissue/ml, wet weight, in 10 mM Tris-maleate containing 110 mM potassium tartrate and 4 mM  $MgCl_2$ , pH 7.4. Samples of 0.2 ml were preincubated at 30° for 15 min in absence or presence of the inhibitor. Uptake was started by adding 50  $\mu$ M [ $^3H$ ]L-glutamate (initial specific activity, 56.0 Ci/mmol; specific activity after isotopic dilution with unlabeled L-glutamate, 0.11 Ci/mmol) with or without 2 mM ATP (to determine nonspecific uptake). The mixture was further incubated for 3 min and the reaction was stopped by adding 2 ml of ice-cold 0.15 M KCl, immediately

followed by filtration through cellulose mixed ester filters (0.65  $\mu$ m pore size; Millipore Corporation, Millipore, Milano, Italy). The radioactivity trapped on the filters was counted in 4 ml of Ultima Gold MV.

## Results

**Synaptosomal [ $^3H$ ]L-Glutamate Uptake.** In our experimental conditions, [ $^3H$ ]L-glutamate uptake in a crude synaptosomal preparation from rat brain cortex follows a simple Michaelis-Menten equation, as indicated by the monophasic inhibition curve obtained in the presence of different concentrations of unlabeled L-glutamate (slope = 1.1) and confirmed by the straight Lineweaver-Burk plot obtained by transforming the same data (Fig. 2A). This indicates a single uptake



**Fig. 2.** [ $^3H$ ]L-glutamate uptake in rat brain cortex synaptosomes. A, raw data of the inhibition curve obtained by coincubating 10  $\mu$ M [ $^3H$ ]L-glutamate with different concentrations of unlabeled L-glutamate (from 5 to 1000  $\mu$ M), in quadruplicate. The inhibition curve was fitted using a "sigmoidal dose-response curve with variable slope" (GraphPad Prism 4.0a) and indicated that the slope (1.1) was not significantly different from 1. The same data were then recalculated taking into account the isotopic dilution and the resulting values (which were well fitted by a rectangular hyperbola with a  $K_d$  value of  $24.6 \pm 2.4$   $\mu$ M) were then transformed for the Lineweaver-Burk plot shown in the inset. B, inhibition curves of HIP-A (▲), HIP-B (▼), TBOA (○), and DHK (◇). For these experiments, synaptosomes were preincubated with the compounds for 7 min, then incubated for 4 min with 10  $\mu$ M [ $^3H$ ]L-glutamate. Nonspecific uptake was measured in the presence of 0.3 mM L-glutamate. The curves were fitted using the "competitive binding, one-site" equation (GraphPad Prism 4.0a). Values are means  $\pm$  S.E.M. from 8 to 12 replications from two-three experiments. Inset, saturation curves obtained by measuring specific [ $^3H$ ]L-glutamate uptake (10–110  $\mu$ M) in the absence (●) or presence of 10  $\mu$ M HIP-A (▲) and HIP-B (▼). HIP-A and HIP-B significantly decreased  $V_{max}$  by 25 and 28%, respectively, with no significant effect on  $K_m$ , indicating noncompetitive inhibition.



mechanism with a  $K_m$  value of  $29 \pm 4 \mu\text{M}$  (mean  $\pm$  S.D. from three experiments), in agreement with previous data (Dambolt, 2001). Thus,  $10 \mu\text{M}$  [ $^3\text{H}$ ]L-glutamate was used for inhibition studies, also because in release experiments (see below) EAAT-mediated heteroexchange was induced by  $10 \mu\text{M}$  L-glutamate. The specific uptake of  $10 \mu\text{M}$  [ $^3\text{H}$ ]L-glutamate is very likely to be mediated only by EAATs, as indicated by the complete  $\text{Na}^+$  dependence (no specific uptake was measured on replacing  $\text{Na}^+$  with  $\text{Li}^+$ ; data not shown), (Kanner and Bendahan, 1982) and by the complete inhibition of specific uptake in the presence of the selective EAAT blocker TBOA (Fig. 2B), showing the expected high affinity ( $\text{IC}_{50}$ ,  $14.5 \mu\text{M}$ ; Table 1). The selective GLT1 inhibitor DHK inhibited [ $^3\text{H}$ ]L-glutamate uptake in a monophasic manner with an  $\text{IC}_{50}$  value of  $386 \mu\text{M}$  (Fig. 2B and Table 1). HIP-A and HIP-B were very potent inhibitors of synaptosomal [ $^3\text{H}$ ]L-glutamate uptake (Fig. 2B), with  $\text{IC}_{50}$  values of 18 and  $17 \mu\text{M}$ , respectively (Table 1). Fig. 2B, inset, shows that, when tested at  $10 \mu\text{M}$ , HIP-A and HIP-B inhibited [ $^3\text{H}$ ]L-glutamate uptake in a noncompetitive manner.

The  $\text{IC}_{50}$  values indicated above were obtained by preincubating the synaptosomes with the drugs for 7 min before adding the [ $^3\text{H}$ ]neurotransmitter. However, we found similar  $\text{IC}_{50}$  for HIP-A on [ $^3\text{H}$ ]L-glutamate uptake with no preincubation ( $24 \mu\text{M}$ ) or after 17 min preincubation ( $13 \mu\text{M}$ ) (Fig. 5A).

HIP-A, HIP-B, and TBOA were also tested on [ $^3\text{H}$ ]D-aspartate uptake where they gave  $\text{IC}_{50}$  values of  $19 \mu\text{M}$  (95% CI, 14–25),  $19 \mu\text{M}$  (95% CI, 17–22), and  $19 \mu\text{M}$  (95% CI, 18–21).

**Vesicular [ $^3\text{H}$ ]L-Glutamate Uptake.** HIP-A and HIP-B, up to  $500 \mu\text{M}$ , had no effect on specific (ATP-dependent) uptake of [ $^3\text{H}$ ]L-glutamate ( $50 \mu\text{M}$ ) in synaptic vesicles prepared from rat forebrain. Trypan Blue was tested in parallel as a positive control, because it was previously characterized as a potent ( $\text{IC}_{50}$ ,  $49 \text{ nM}$ ), selective, and competitive inhibitor of vesicular glutamate transporters (Roeth et al., 1998). In agreement, we found that Trypan Blue potently inhibited [ $^3\text{H}$ ]L-glutamate uptake in synaptic vesicles with an  $\text{IC}_{50}$  of  $137 \text{ nM}$ .

**[ $^3\text{H}$ ]D-Aspartate Release from Superfused Synaptosomes.** For release experiments, we preloaded synaptosomes with [ $^3\text{H}$ ]D-aspartate and stratified them in a superfusion apparatus. After 24 min of superfusion (equilibration period), the synaptosomes present in each superfusion chamber contained  $151,900 \pm 5800 \text{ dpm}$  (mean  $\pm$  S.E.M.,  $n = 25$  experiments) and released about 2% of this radioactivity in the collected 2-min fractions (basal release; Fig. 3, inset).

Exposure of superfused synaptosomes to L-glutamate (the

prototypical EAAT substrate) increased [ $^3\text{H}$ ]D-aspartate release. This was concentration-dependent, small at 1 to  $3 \mu\text{M}$  but rising steeply from 10 to  $100 \mu\text{M}$  (Fig. 3). We confirmed that exposure of superfused synaptosomes to nontransportable EAAT blockers (TBOA and DHK) had no effect on basal [ $^3\text{H}$ ]D-aspartate release (Fig. 3). HIP-A and HIP-B induced some [ $^3\text{H}$ ]D-aspartate release at concentrations  $\geq 3 \mu\text{M}$ , although much lower than that induced by L-glutamate (Fig. 3).

**L-Glutamate-Induced [ $^3\text{H}$ ]D-Aspartate Release.** The [ $^3\text{H}$ ]D-aspartate release induced by  $10 \mu\text{M}$  L-glutamate was similar, with superfusion buffer containing  $\text{Ca}^{2+}$  ions or not (in 4 min:  $9.17 \pm 0.89\%$  versus  $9.67 \pm 0.83\%$ , mean  $\pm$  S.D.,  $n = 4$ ), indicating a nonexocytotic release. It was dose-dependently inhibited by pre-exposing synaptosomes to TBOA (7 min before L-glutamate) with an  $\text{IC}_{50}$  of about  $7 \mu\text{M}$  and a nearly complete inhibition at  $61 \mu\text{M}$  (Fig. 4 and Table 1). DHK also inhibited the L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release with an  $\text{IC}_{50}$  of  $235 \mu\text{M}$  (Fig. 4 and Table 1).

HIP-A and HIP-B were not tested at concentrations higher than  $3 \mu\text{M}$  because of their releasing effects (see Fig. 4). However, exposure of synaptosomes (7 min before L-glutamate) to lower concentrations of HIP-A and HIP-B ( $1$ – $3 \mu\text{M}$ ) markedly and significantly inhibited (by 35–70%) the L-glutamate-induced release (Fig. 4), with  $\text{IC}_{50}$  values of 1.6 and  $1.2 \mu\text{M}$  (Table 1). It should be noted that the same low concentrations of HIP-A and HIP-B had very little effect (less than 10%) on [ $^3\text{H}$ ]L-glutamate uptake (Fig. 2).

Figure 5B shows that the inhibitory effect of  $1.75 \mu\text{M}$  HIP-A on L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release was only evident when the drug was added in the superfusion medium 7 to 17 min before L-glutamate (60 and 74% inhibition, respectively). If added concomitantly to L-glutamate, it had no inhibitory effect. As shown in Fig. 5A, the same preincubation conditions did not significantly modify the inhibitory effect of HIP-A on [ $^3\text{H}$ ]L-glutamate uptake. Thus,  $1.75 \mu\text{M}$  HIP-A inhibited specific uptake by 7% when added concomitantly with [ $^3\text{H}$ ]L-glutamate and by 12% when added 17 min before.

**[ $^3\text{H}$ ]D-Aspartate Release Induced by Reversal of the Ion Gradients.** EAAT-mediated [ $^3\text{H}$ ]D-aspartate release could be induced in superfused synaptosomes by other experimental conditions, which affect the ion gradients by different mechanisms and consequently favor the reversal of the transporter function. In particular, we inhibited the energy-dependent processes by adding rotenone and 2-deoxy-glucose in glucose-free medium (Fig. 6, A and B); we increased the  $\text{K}^+$  concentration of the superfusion buffer with a concomitant

TABLE 1

Comparison between the  $\text{IC}_{50}$  values of HIP-A, HIP-B, TBOA, and DHK as inhibitors of L-glutamate uptake and of L-glutamate-induced release. For both assays, rat brain cortex synaptosomes were prepared in the same way and resuspended in the same buffer; they were preincubated for 7 min with drugs and then exposed for 4 to 8 min to the same concentration of L-glutamate ( $10 \mu\text{M}$ ) to measure either the uptake of [ $^3\text{H}$ ]L-glutamate (Fig. 2) or L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release (Fig. 4). The inhibition curves (four to six concentrations of the compounds, from at least three independent experiments) were fitted using the 'one-site competition' equation built into GraphPad Prism, which gives the  $\text{IC}_{50}$  with the 95% CI in brackets. The ratio between the  $\text{IC}_{50}$  values measured in the two assays is shown in the last column.

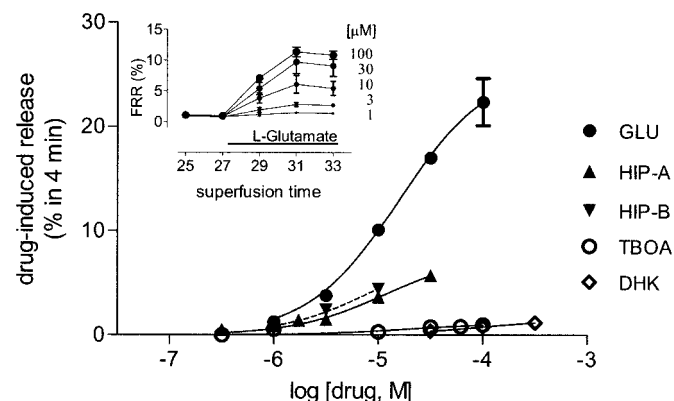
	[ $^3\text{H}$ ]L-Glutamate Uptake	L-Glutamate-Induced [ $^3\text{H}$ ]D-Aspartate Release	Ratio $\text{IC}_{50}$ Uptake/Release
	$\mu\text{M}$	$\mu\text{M}$	
HIP-A	18.0(14.7–21.9)	1.6(1.2–2.1)	11.3
HIP-B	16.9(14.8–19.2)	1.2(0.8–1.8)	14.1
TBOA	14.5(12.9–16.5)	7.3(5.4–9.9)	2.0
DHK	386 (328–453)	235 (141–389)	1.6

decrease of  $\text{Na}^+$  concentration (Fig. 6C), and we increased the intracellular  $\text{Na}^+$  concentration by using the  $\text{Na}^+/\text{H}^+$  ionophore monensin (Fig. 6D). The  $[^3\text{H}]\text{D}$ -aspartate release induced by rotenone/2-deoxy-glucose was completely inhibited by 100  $\mu\text{M}$  TBOA, suggesting that it is not caused by

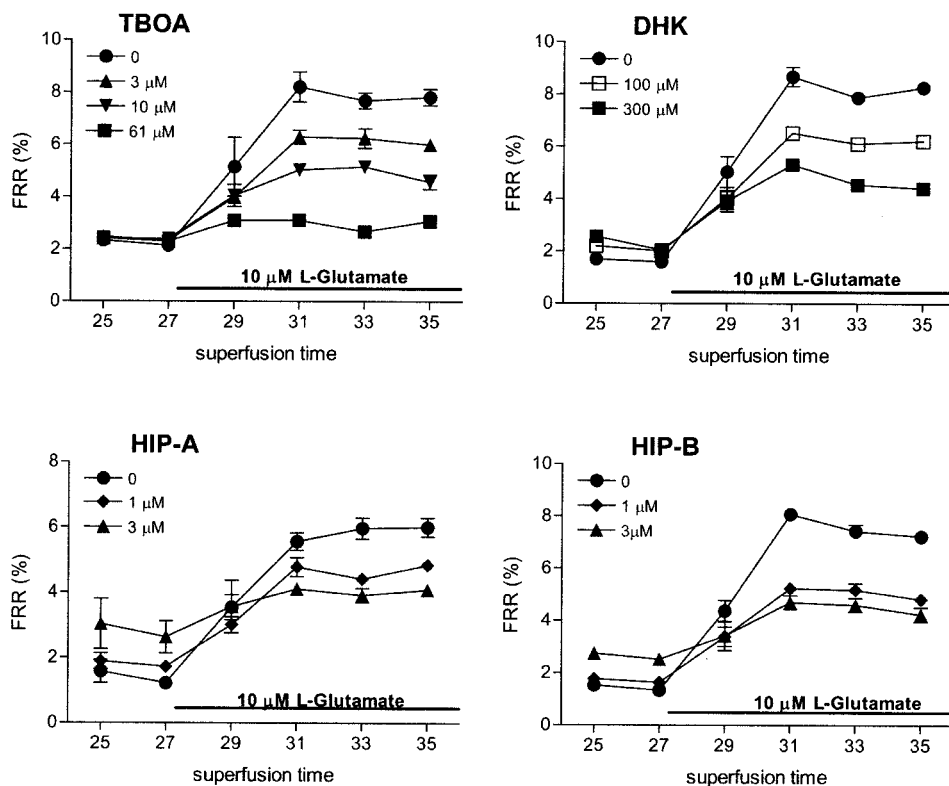
nonspecific effects on synaptosomal membrane integrity and that it is mainly fully EAAT-mediated. On the contrary, only about 50% of the release induced by both high  $\text{K}^+$  and monensin is EAAT-mediated, as indicated by the partial effect observed with a saturating TBOA concentration (100  $\mu\text{M}$ ). It should be noted, however, that 10  $\mu\text{M}$  TBOA inhibited the EAAT-mediated release induced by high  $\text{K}^+$  by more than 50% (Fig. 6C), indicating that the potency of TBOA in this model is similar to its potency on the glutamate-induced release ( $\text{IC}_{50} = 7 \mu\text{M}$ ). On the contrary, Fig. 6 shows that 1.75  $\mu\text{M}$  of HIP-A and HIP-B; i.e., the concentration that inhibited the glutamate-induced release by  $\geq 50\%$  had no effect on the  $[^3\text{H}]\text{D}$ -aspartate release induced by all the conditions reversing the ion gradients.

## Discussion

HIP-A and HIP-B were potent and noncompetitive inhibitors of synaptosomal  $[^3\text{H}]\text{L}$ -glutamate uptake, with  $\text{IC}_{50}$  values (17–18  $\mu\text{M}$ ) very similar to that of the potent and competitive EAAT inhibitor TBOA (Shimamoto et al., 1998). Release experiments further suggested that both HIP-A and HIP-B behave as EAAT blockers and not as substrates. Thus, the two compounds have a very limited effect in inducing  $[^3\text{H}]\text{D}$ -aspartate release compared with the marked releasing effect of the prototypical substrate L-glutamate, but they potentially inhibited L-glutamate-induced  $[^3\text{H}]\text{D}$ -aspartate release, as did TBOA. The complete inhibition with TBOA confirms that, in our experimental model of superfused synaptosomes, L-glutamate-induced  $[^3\text{H}]\text{D}$ -aspartate release is mediated by EAATs, possibly by heteroexchange and that this model is useful for studying nontransportable EAAT blockers, as suggested previously (Koch et al., 1999b). The selective GLT1 blocker DHK (Arriza et al., 1994) inhibited



**Fig. 3.** Concentration-effect curves for drug-induced  $[^3\text{H}]\text{D}$ -aspartate release from superfused rat brain cortex synaptosomes. The drug-induced release was calculated from data such as those shown, for example, in the inset, regarding the release induced by increasing concentrations of L-glutamate (1–100  $\mu\text{M}$ ). For these experiments, synaptosomes were exposed to the drugs after 27 min of superfusion (equilibration period). Five 2-min fractions were collected from  $t = 24$  to  $t = 34$  min (in the inset, the value corresponding to the fraction from  $t = 24$  to  $t = 26$  is indicated at  $t = 25$  and so on). The radioactivity in each fraction was calculated as a percentage of the total radioactivity in the synaptosomes at the start of that fraction and is referred to as FRR (see inset). The drugs-induced release shown in the main graph was calculated by subtracting the sum of FRR values in the two fractions preceding drug exposure ( $t = 24$ – $26$  and  $t = 26$ – $28$ ) from the sum of FRR values measured in the two fractions after drug exposure ( $t = 30$ – $32$  and  $t = 32$ – $34$ ). It should be noted that the effect of the drugs added at  $t = 27$  min was only detectable 1.5 min later (i.e., the time required to the fluid to flow from the filters). Each value is the mean  $\pm$  S.D. of three to nine replications.

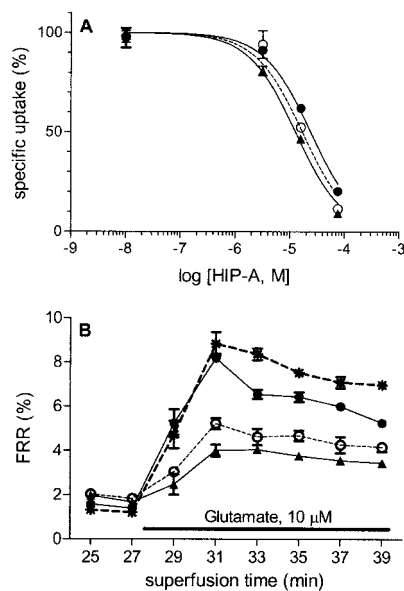


**Fig. 4.** Concentration-dependent antagonism by TBOA, DHK, HIP-A, and HIP-B of the  $[^3\text{H}]\text{D}$ -aspartate release induced by 10  $\mu\text{M}$  glutamate from rat brain cortex synaptosomes. Superfused synaptosomes were pre-exposed to antagonists from  $t = 20$  min, and L-glutamate (10  $\mu\text{M}$ ) was added in the superfusion buffer from  $t = 27$  min. Six 2-min fractions were collected from  $t = 24$  on. The FRR was calculated as 100 times the amount of radioactivity released into each 2-min fraction over the total radioactivity present on the filter at the start of that fraction. Each graph shows the data from one representative experiment, each point indicating the mean  $\pm$  S.D. of the FRR values measured in four or five superfusion chambers. Note that the value corresponding to the fraction from  $t = 24$ – $26$  is indicated at  $t = 25$ , and so on, and that the effect of the drugs added at  $t = 27$  was only detectable 1.5 min later (i.e., the time required to the fluid to flow from the filters to the collecting vials). The glutamate-induced release could be calculated by subtracting the sum of FRR values in the two fractions preceding glutamate exposure ( $t = 24$ – $26$  and  $t = 26$ – $28$ ) from the sum of FRR values measured in the two fractions after glutamate exposure ( $t = 30$ – $32$  and  $t = 32$ – $34$ ). The comparison of the glutamate-induced release measured in the absence or presence of the compounds allowed us to calculate their inhibitory effects ( $\text{IC}_{50}$  values reported in Table 1).

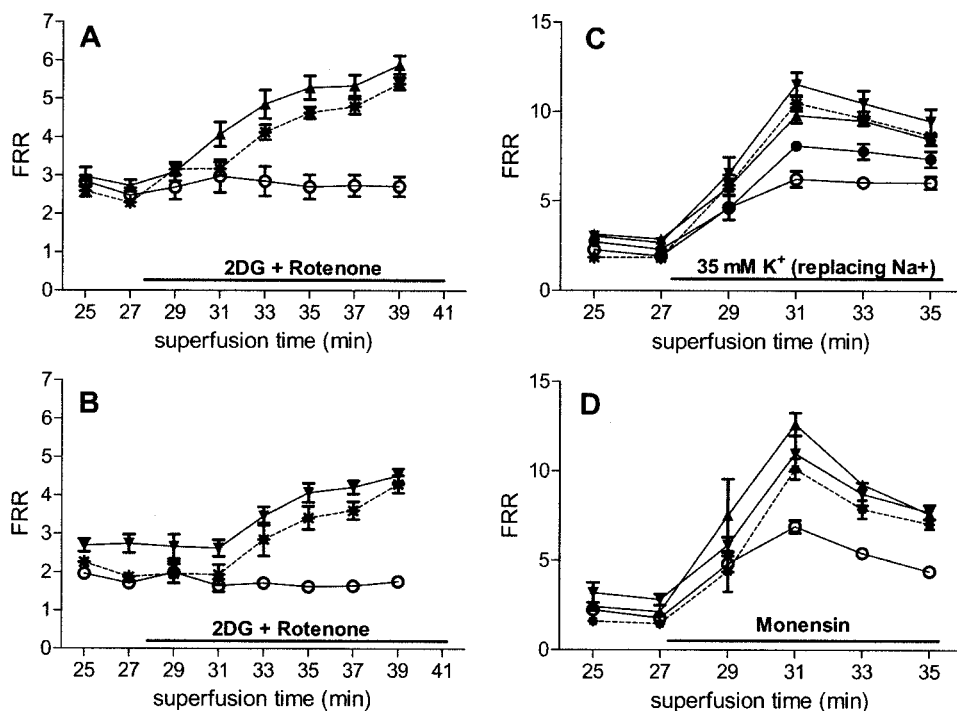
synaptosomal [ $^3\text{H}$ ]L-glutamate uptake with an  $\text{IC}_{50}$  of 386  $\mu\text{M}$ , which is much higher than the corresponding value described on recombinant human EAAT<sub>2</sub> (23  $\mu\text{M}$ ; Arriza et

al., 1994) but is not very different from some of the values found in rat cortical synaptosomes ( $\geq 100 \mu\text{M}$ ; Robinson et al., 1991; Garlin et al., 1995; Dowd et al., 1996). Note that very similar  $\text{IC}_{50}$  values were found using three different batches of DHK from two commercial sources. Most importantly, DHK did inhibit glutamate-induced [ $^3\text{H}$ ]D-aspartate release with a very similar  $\text{IC}_{50}$  (230  $\mu\text{M}$ ), suggesting that both glutamate uptake and glutamate-induced release are mediated by GLT1-like transporters, in agreement with the purported pharmacology of synaptosomal EAATs (Tanaka et al., 1997; Koch et al., 1999a; Suchak et al., 2003).

The main finding of the present study, however, was that the concentrations of HIP-A and HIP-B required to inhibit L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release ( $\text{IC}_{50}$  1.6  $\mu\text{M}$ ) were more than 10 times lower than those required to inhibit [ $^3\text{H}$ ]L-glutamate uptake. Therefore, there was a concentration window (1–3  $\mu\text{M}$ ) in which the two compounds significantly inhibited L-glutamate-induced release with very little effect (less than 10%) on L-glutamate uptake. It is very unlikely that this difference is an artifact arising from the comparison of two assays with different intrinsic characteristics. Thus, 1) uptake and release experiments were done in conditions as similar as possible. For both assays, synaptosomes were prepared the same way and resuspended in the same buffer, they were preincubated for 7 min with drugs and then exposed for 4 to 8 min to the same concentration of L-glutamate (10  $\mu\text{M}$ ) to measure either the uptake of [ $^3\text{H}$ ]L-glutamate or L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release; 2) the marked difference found with HIP-A and HIP-B was not seen with TBOA and DHK, which were only twice as potent in inhibiting L-glutamate-induced release than [ $^3\text{H}$ ]L-glutamate uptake; 3) the preincubation time of synaptosomes with HIP-A affected the effect on L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release but not [ $^3\text{H}$ ]L-glutamate uptake. This last observation rules out that a small inhibition of L-glutamate



**Fig. 5.** Effect of different pre-exposure times of synaptosomes to HIP-A on its ability to inhibit synaptosomal uptake of 10  $\mu\text{M}$  [ $^3\text{H}$ ]L-glutamate (A) or to antagonize the [ $^3\text{H}$ ]D-aspartate release induced by 10  $\mu\text{M}$  L-glutamate (B). For the uptake experiments (A), rat brain cortex synaptosomes were preincubated in the absence or presence of the HIP-A for 0 (●), 7 (○) and 17 min (▲), then incubated for 4 min with 10  $\mu\text{M}$  [ $^3\text{H}$ ]L-glutamate. Nonspecific uptake was measured in the presence of 300  $\mu\text{M}$  L-glutamate. Each value shows the specific uptake as percentage of the value obtained without HIP-A and is the mean  $\pm$  S.D. of four replications. Inhibition curves were fitted using the “one-site competition” equation built into GraphPad Prism. For the release experiments (B), superfused brain cortex synaptosomes preloaded with [ $^3\text{H}$ ]D-aspartate were pre-exposed to 1.75  $\mu\text{M}$  HIP-A at 17 (▲), 7 (○) and 0 min (●) before exposure to 10  $\mu\text{M}$  L-glutamate (added in the superfusion medium as indicated). Asterisks show the release induced by glutamate in the absence of HIP-A. Each value is the mean  $\pm$  S.D. of five replications.



**Fig. 6.** [ $^3\text{H}$ ]D-aspartate release induced from rat brain cortex synaptosomes by different experimental conditions favoring reverse transport, in the absence (\*) or presence of 1.75  $\mu\text{M}$  HIP-A (▲), 1.75  $\mu\text{M}$  HIP-B (▼), and 10  $\mu\text{M}$  (●) or 100  $\mu\text{M}$  (○) TBOA. Superfused synaptosomes were pre-exposed to drugs from  $t = 20$  min, and from  $t = 27$  min, synaptosomes were exposed to the following releasing conditions: A and B, a glucose-free superfusion buffer containing 2 mM deoxyglucose (DG) and 5  $\mu\text{M}$  rotenone, leading to inhibition of energy-dependent processes; C, a superfusion buffer in which the KCl concentration was raised from 5 to 35 mM (replacing an equimolar concentration of NaCl); D, in the presence of 3  $\mu\text{M}$  monensin, a  $\text{Na}^+/\text{H}^+$  ionophore. Each graph shows the results of a representative experiment; data points represent mean  $\pm$  S.D. of four or five replications.



uptake is sufficient, on its own, to markedly reduce L-glutamate-induced release.

It also seems unlikely that the preferential effect of HIP-A and HIP-B in inhibiting L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release rather than [ $^3\text{H}$ ]L-glutamate uptake might be caused by greater affinity for hypothetical "aspartate-prefering sites" on glutamate transporters, because their  $\text{IC}_{50}$  values on [ $^3\text{H}$ ]D-aspartate and [ $^3\text{H}$ ]L-glutamate uptake were identical; moreover, HIP-A is an aspartate analog, whereas HIP-B contains the skeleton of glutamic acid.

Another possibility was that even at the lowest concentrations (1–3  $\mu\text{M}$ ), HIP-A and HIP-B might enter the synaptosomes with an EAAT-independent mechanism, reaching sufficient intracellular concentrations (i.e.,  $\geq 10 \mu\text{M}$ ) to inhibit the intracellular interaction of [ $^3\text{H}$ ]D-aspartate with EAATs. This, too is unlikely, however, because 1 to 3  $\mu\text{M}$  HIP-A and HIP-B had no effect on the EAAT-mediated [ $^3\text{H}$ ]D-aspartate reverse transport induced by experimental conditions altering, by different mechanisms, the ion gradients required for the inward transport (note that in these conditions, too, HIP-A and HIP-B were added in the superfusion medium 7 min before the releasing stimuli). The lack of effect in these experimental models also presumably excludes that HIP-A and HIP-B inhibited glutamate-induced [ $^3\text{H}$ ]D-aspartate release by acting as agonists on an as-yet-unidentified "inhibitory" receptor.

It seems, therefore, that low HIP-A and HIP-B concentrations specifically affect some glutamate-triggered process(es)—different from glutamate translocation itself—responsible for the induction of EAAT-mediated [ $^3\text{H}$ ]D-aspartate reverse transport.

A first possibility is that L-glutamate-induced release involves, at least in part, its interaction with targets other than EAATs (e.g., glutamate receptors or vesicular glutamate transporters) sensitive to low concentrations of HIP-A/B, also resulting in EAAT-mediated (TBOA-sensitive) release. The only glutamate receptor showing some affinity for HIP-A and HIP-B are AMPA ( $\text{IC}_{50}$ , 43 and 35  $\mu\text{M}$ ) and kainate ( $\text{IC}_{50}$ , 8 and 45  $\mu\text{M}$ ) receptors (Conti et al., 1999b) but an involvement of these receptors is unlikely. Thus, 4,5,6,6a-tetrahydro-3aH-pyrrolo[3,4-d]isoxazole-3,4-dicarboxylic acid (Fig. 1), the carboxy analog of HIP-A, which is a high-affinity ligand for AMPA ( $\text{IC}_{50}$  1.3  $\mu\text{M}$ ) and kainate ( $\text{IC}_{50}$  0.5  $\mu\text{M}$ ) receptors (Conti et al., 1999b), had no effect on basal or L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release (data not shown). Furthermore, HIP-A and HIP-B do not affect vesicular glutamate uptake, indicating that they do not interact with vesicular transporters.

Another theoretical possibility is that glutamate uptake and glutamate-induced release are mediated by different transporters, both of which, however, are DHK-sensitive. Although two GLT1 variants have been described (Chen et al., 2002), we consider it unlikely that one of them (i.e., the one that is insensitive to the lowest HIP concentrations and accounts for most of [ $^3\text{H}$ ]glutamate uptake) could work in only one direction. The final possibility is that the releasing effect of L-glutamate, although depending on its interaction with EAATs, is dissociated from EAAT-mediated glutamate uptake, considering the sensitivity to low concentrations of HIP-A and HIP-B.

These data do not adhere to the classic model of facilitated exchange-diffusion (Fischer and Cho, 1979), which assumes

that the transporter-mediated release of intracellularly located substrates is strictly coupled to the transporter-mediated influx (i.e., uptake) of extracellular substrates. The dissociation between the two processes could be assumed from previous data obtained using synaptic plasma membrane vesicles from rat brain showing that  $\text{Li}^+$  can replace  $\text{Na}^+$  for glutamate-induced efflux but not for glutamate influx (Kanner and Bendahan, 1982). However, we did not replicate this finding because in our hands  $\text{Li}^+$  could not act in place of  $\text{Na}^+$  for L-glutamate-induced [ $^3\text{H}$ ]D-aspartate release. The dissociation between substrate uptake and substrate-induced release was previously suggested for dopamine transporters (DATs), on the basis of data showing that zinc ions *inhibited* [ $^3\text{H}$ ]dopamine uptake but *enhanced* amphetamine-induced dopamine transporter-mediated release (Scholze et al., 2002) and might therefore be a general feature of transporters. It was interesting that only amphetamine-induced reverse transport, but not reverse transport induced by lowering extracellular sodium, is stimulated by  $\text{Zn}^{2+}$  and is accompanied by uncoupled  $\text{Na}^+$  currents also stimulated by  $\text{Zn}^{2+}$  (Piffl et al., 2004). These data suggested a link between amphetamine-induced inward currents and amphetamine-induced reverse transport (Piffl et al., 2004).

The available data on EAATs suggest that glutamate translocation can be dissociated from glutamate-induced, EAAT-mediated, uncoupled  $\text{Cl}^-$  currents (Wadiche et al., 1995; Wadiche and Kavanaugh, 1998; Seal et al., 2001; Borre et al., 2002; Ryan and Vandenberg, 2002). Our present data indicate that glutamate-induced reverse transport (sensitive to low concentrations of HIP-A and HIP-B) represents a process independent from glutamate influx (insensitive to the two compounds); therefore, it is tempting to speculate that it might require the substrate-induced uncoupled currents. It is important to note that HIP-A and HIP-B do not modify the reverse transport induced by conditions affecting the ion gradients. It is also very interesting that the inhibition of [ $^3\text{H}$ ]L-glutamate uptake shown by a higher HIP-A and HIP-B concentration (10  $\mu\text{M}$ ) is noncompetitive, suggesting that the two compounds bind to a site different from the one responsible for glutamate inward transport. Compounds such as HIP-A and HIP-B, or compounds with an even better selectivity, could be very useful to further clarify the mechanisms underlying these operating modes of transporters.

Finally, the therapeutic value of HIP-A and HIP-B in counteracting ischemia-induced neuronal degeneration would be worth investigating. Although HIP-A and HIP-B will not affect the glutamate release induced by the alteration of the ion gradients, they could inhibit the consequent glutamate-induced glutamate release whose importance in ischemic damage has yet to be established.

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