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L-*TRANS*-PYRROLIDINE-2,4-DICARBOXYLATE AND *CIS*-1-AMINOCYCLOBUTANE-1,3-DICARBOXYLATE BEHAVE AS TRANSPORTABLE, COMPETITIVE INHIBITORS OF THE HIGH-AFFINITY GLUTAMATE TRANSPORTERS

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Abstract—The ability of two conformationally restricted analogues of L-glutamate to function as non-transportable inhibitors of plasma membrane L-glutamate transport was investigated in primary cultures of cerebellar granule cells and cortical astrocytes. L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (L-*trans*-PDC) and *cis*-1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD) behaved as linear competitive inhibitors of the uptake of D-[^{3}H]aspartate (used as a non-metabolizable analogue of L-glutamate) exhibiting K_i values between 40 and 145 μM; L-*trans*-PDC being the more potent inhibitor in each preparation. However, both L-*trans*-PDC and *cis*-ACBD, over a concentration range of 1 μM–5 mM, dose-dependently stimulated the release of exogenously supplied D-[^{3}H]aspartate from granule cells maintained in a continuous superfusion system. The stimulated release was independent of extracellular calcium ions; essentially superimposable dose–response profiles being obtained in the absence and presence of 1.3 mM $CaCl_2$ and yielding EC_{50} values of 16–25 μM and 180–220 μM for L-*trans*-PDC and *cis*-ACBD, respectively. Stimulated release of D-[^{3}H]aspartate was unaffected by either 300 μM D-(−)-2-amino-5-phosphonopentanoic acid [D-APV; a selective antagonist of the *N*-methyl-D-aspartate (NMDA) receptor] or by 25 μM 6-cyano-7-nitroquinoxaline-2,3-dione [CNQX; a selective antagonist of the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor]. The release of D-[^{3}H]-aspartate following stimulation by either L-*trans*-PDC or *cis*-ACBD was however markedly attenuated following substitution in the superfusion medium of sodium ions by choline ions. Taken together, these results support an action of L-*trans*-PDC and *cis*-ACBD consistent with that of being competitive substrates rather than non-transportable blockers of the plasma membrane L-glutamate uptake system.

The central role played by L-glutamate (Glu‡) in normal mental processes such as learning and memory, in brain damage following anoxic/ischaemic insult and in a variety of idiopathic and inherited neurodegenerative disorders is now well-established [1]. The actions of Glu both as an excitatory amino acid (EAA) neurotransmitter and as a neurotoxin are, paradoxically, mediated by activation of the same receptor subtypes; namely the ionotropic [*N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and kainate (KA)] receptors [2] and metabotropic receptors [3]. The development of numerous agonists and antagonists for the various EAA receptor subtypes has contributed greatly to an understanding of the structural requirements of Glu binding to EAA receptor recognition sites. In contrast, relatively little is known about the structural requirements of binding to the high-affinity pre-synaptic plasma membrane Glu transporter [4–8], although the recent cloning of at least three distinct cDNAs encoding structurally related EAA (Glu) transporters [9–11] should lead to a better understanding of the molecular aspects of transporter structure, function and ligand binding characteristics. Under normal circumstances, transport systems located in both neurons and astrocytes [12] terminate the post-synaptic actions of Glu by effecting its rapid removal from the synapse. A number of findings have served to focus interest on the functional characteristics of the pre-synaptic Glu transporters and in the design of compounds with which they might interact selectively: (i) Glu release from brain cells via reversal of the transporter may be implicated in the pathogenesis of certain neurodegenerative conditions [13–16], (ii) *in vitro* studies using neuronal and astrocytic cultures indicate that Glu transport is an effective mechanism for preventing or limiting excitotoxic cell death [17–20] and (iii) the pathophysiology of certain neurodegenerative disorders, e.g. amyotrophic lateral sclerosis and Alzheimer's disease, may involve a specific defect in either the rate of Glu transport [21] or in its binding to the transporter [22].

The development of "non-transportable" inhibi-

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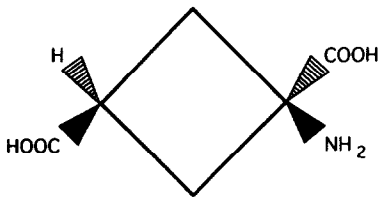
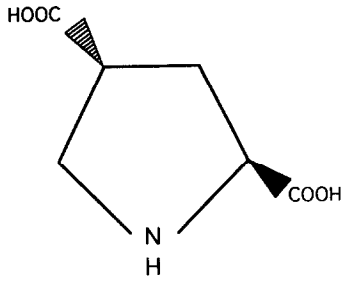
‡ Abbreviations: L-*trans*-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; *cis*-ACBD, *cis*-1-aminocyclobutane-1,3-dicarboxylic acid; Glu, L-glutamate; NMDA, *N*-methyl-D-aspartate; D-APV, D-(−)-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; EAA, excitatory amino acid; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline.



cis -1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD)



L-*trans* -pyrrolidine-2,4-dicarboxylic acid (L-*trans* -PDC)

Fig. 1. Chemical structures of L-*trans*-PDC and *cis*-ACBD.

tors of the Glu transporter for use as selective tools in studying the mechanism(s) of Glu release would be advantageous compared to "transportable" inhibitors since the latter might be expected to evoke Glu release via carrier reversal. This would serve only to complicate the interpretation of experimental observations. Although a number of compounds are available which inhibit the Glu transport system, none appear to combine high potency with non-transportability. Moreover, a number of Glu uptake inhibitors, e.g. dihydrokainate together with the L- and D-enantiomers of threo-β-hydroxyaspartate and also of the acidic sulphur-containing amino acids, may also exhibit appreciable affinity for EAA receptors [7, 23–27]. More recently, Glu uptake blockers have been developed which exhibit little or no interaction with ionotropic EAA receptors [8, 28, 29]. Two compounds of particular interest are L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-PDC) [29] and *cis*-1-aminocyclobutane-1,3-dicarboxylic acid (*cis*-ACBD) [28, 30]. The structures of these compounds are presented in Fig. 1. The present investigation was undertaken (i) to ascertain whether L-*trans*-PDC and/or *cis*-ACBD are substrates for the high-affinity Glu transporter, and (ii) to characterize the nature of their inhibitory effects on high-affinity Glu uptake in primary cultures of neurons and astrocytes, and moreover, (iii) to evaluate any selective action of the inhibitors on neuronal versus glial transport. In the absence of any direct method for measuring L-*trans*-PDC and *cis*-ACBD uptake, the strategy adopted was that of attempting to demonstrate that both compounds were competitive inhibitors of the Glu carrier, and in addition, to show that both were able to evoke the release, by calcium-independent carrier reversal, of previously accumulated D-[^{3}H]aspartate (used as a non-metabolizable analogue of Glu) from primary cultures of cerebellar granule "glutamatergic" cells used as an appropriate *in vitro* model system.

MATERIALS AND METHODS

Materials. Plastic 24-well multidishes and 35-mm Petri dishes, and Dulbecco's minimum essential medium (DMEM) were purchased from Gibco/Life Technologies (U.K.). Dibutyryl-cyclic AMP (dBcAMP), cytosine arabinoside, trypsin, penicillin, insulin, soybean trypsin inhibitor and poly-L-lysine were obtained from the Sigma Chemical Co. (Poole, U.K.). Foetal calf serum was purchased from Sera-Lab Ltd (Sussex, U.K.). L-*trans*-Pyrrolidine-2,4-dicarboxylic acid (L-*trans*-PDC), *cis*-1-amino-cyclobutane-1,3-dicarboxylic acid (*cis*-ACBD), D-(−)-2-amino-5-phosphonopentanoic acid (D-APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Tocris Neuramin (Bristol, U.K.). D-[^{3}H]Aspartic acid (sp. act. 15 Ci/mmol) was obtained from Du Pont (UK) Ltd (Herts, U.K.).

Cell culture. Cerebellar granule cells were cultured essentially as described by Schousboe *et al.* [31]. Briefly, cerebella from 7-day-old CD1 mice (obtained from the animal quarters of the University of St Andrews) were dissociated by mild trypsinization [0.1% (w/v) trypsin at 37° for 15 min] and subsequently inoculated into poly-L-lysine-coated 24-well multi-dishes (for uptake experiments) at a cell density of 4.5×10^5/well or 35-mm Petri dishes (for release experiments) at a cell density of 4.5×10^6/dish, following resuspension in slightly modified DMEM (24.5 mM KCl, 30 mM glucose) supplemented with *p*-aminobenzoate (7 μM), insulin (100 mU/L) and 10% (v/v) foetal calf serum. Cells were maintained in culture for 7–10 days, with the addition of the antimitotic agent cytosine arabinoside (40 μM) from day 2 *in vitro* to prevent glial proliferation.

Astrocytes were cultured essentially as previously described by Hertz *et al.* [32]. Briefly, prefrontal cortex was taken from newborn mice and passed through sterile nylon sieves (80 μm pore size) into a modified Eagle's minimum essential medium containing 20% (v/v) inactivated foetal calf serum and inoculated into the individual wells of NUNCLON multitest dishes. The cultures were grown for a total of 3 weeks changing the culture medium 2 days after inoculation and subsequently twice a week. During the last week of cultivation the serum concentration was reduced to 10% (v/v) and 0.25 mM dBcAMP was added to the culture medium. Such cultures consist of well-differentiated astrocytes and have been shown to be devoid of neurons.

Uptake assays. Uptake of D-aspartate was performed as detailed elsewhere [7]. Briefly, for assays employing both cerebellar granule cells and astrocytes, the culture medium was exchanged with phosphate-buffered saline (PBS; 135 mM NaCl, 3.0 mM KCl, 1.0 mM $CaCl_2$, 0.6 mM $MgSO_4$, 1.7 mM KH_2PO_4, 8.0 mM Na_2HPO_4, and 6.0 mM glucose, pH 7.4) containing D-aspartate at the desired

concentrations, and the cells preincubated for 3 min. Subsequently, this medium was exchanged with PBS containing different concentrations of the ^{3}H-labelled D-aspartate (range 5–5000 μM) and incubated for 3 min. Thereafter, the medium was removed by rapid aspiration and excess radioactivity removed by further aspiration (within 2 sec) following washing with non-radioactive medium. To determine entrapped radioactivity, some incubations were performed at 0° for 2 sec. Cells were dissolved in 0.1 mL of 2 N KOH and samples taken for measurements of radioactivity (by liquid scintillation counting) and for protein content using the method of Lowry *et al.* [33]. The inhibitory effect of L-*trans*-PDC and *cis*-ACBD was studied at inhibitor concentrations specified in the text and figure legends.

Measurement of release. Release of D-[^{3}H]-aspartate was measured essentially as described by Drejer *et al.* [34] and Dunlop *et al.* [35]. Cerebellar granule cells were pre-loaded for 30 min at 37° in the presence of 1 μM D-[^{3}H]aspartate (2.5 μCi/culture). Following preincubation, the cells were overlaid with 1 mL HEPES-buffered saline (HBS) comprising 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 1.0 mM $CaCl_2$, 6.0 mM glucose, pH 7.4. The cells were covered with a nylon mesh (80 μm mesh size) to facilitate dispersion of the medium over the cell monolayer and then placed on a superfusion system. This system comprises a peristaltic pump continuously delivering superfusion medium (37°) at a flow rate of 2 mL/min from a reservoir to the top of a slightly tilted Petri dish. The medium was continuously collected from the lower part of the dish and delivered to a fraction collector. Following washout of excess radioactivity, the cells were subsequently stimulated for 1 min every 4 min by changing the superfusion medium from HBS to a corresponding medium supplemented with either (i) L-*trans*-PDC or *cis*-ACBD alone; (ii) L-*trans*-PDC or *cis*-ACBD plus EAA receptor antagonist. In experiments undertaken to evaluate ionic requirements, changes were made to the HBS medium: (i) the effect of calcium ions on release was studied by replacing $CaCl_2$ with an equimolar concentration of $CoCl_2$, and (ii) for sodium ion substitution experiments the extracellular NaCl was replaced by an equimolar concentration of choline chloride. Fractions (1 mL) were collected at 30 sec intervals and the radioactivity in each fraction was determined by liquid scintillation counting following addition of 3 mL Ecoscint A (National Diagnostics, U.K.) to each 1 mL fraction.

Data analysis. The kinetic parameters, K_m and V_{max}, for uptake were calculated by weighted, non-linear regression analysis using the ENZFITTER software program [36]. Neuronal and astrocytic uptake was found to best fit the Michaelis–Menten equation which comprised an additional component for non-saturable influx [37]. EC_{50} values were determined using the Fit software package [38]. The inhibition constants (K_i) were calculated using the equation for linear competitive inhibition [39].

RESULTS

Kinetic analysis of transport and effect of inhibitors

Experimental conditions were first established for measuring accurately the kinetics of D-aspartate transport in cultures of granule cells and astrocytes. Following subtraction of zero time controls to correct for extracellular trapping, the uptake of D-aspartate could be resolved into an apparent non-saturable component and a saturable, carrier-mediated transport (not shown). In the presence of inhibitor (i.e. L-*trans*-PDC or *cis*-ACBD) only the carrier-mediated uptake of D-aspartate was inhibited, the non-saturable component being essentially unaffected.

In view of a significant contribution of non-saturable influx to the total uptake of D-aspartate, particularly at higher substrate concentrations and in the presence of inhibitor, it was important that data analysis determined the effect of uptake inhibitors only on the saturable component of uptake. In many studies the non-saturable uptake is assessed and subtracted from total uptake using parallel incubations at 4°. This may not however be sufficient since this component of uptake may also be temperature sensitive. Thus, uptake of D-aspartate was measured in the absence and presence of uptake inhibitors using a sufficiently wide range (1–5000 μM) of substrate concentration such that non-saturable uptake could be accurately determined.

The kinetic parameters, K_m and V_{max}, for carrier-mediated uptake of D-aspartate and the effects of L-*trans*-PDC and *cis*-ACBD on these parameters are summarized in Tables 1 and 2. D-aspartate exhibits appreciably higher affinity as a substrate of the Glu transporter in cerebellar granule cells (K_m = 16–28 μM) compared to that in astrocytes (K_m = 51–64 μM); whereas in both cell types the V_{max} values are essentially similar. To obtain information about the mode of action and inhibitor potency of L-*trans*-PDC and *cis*-ACBD, inhibition kinetic studies were undertaken in the presence of two different concentrations of each inhibitor. Computer-assisted

Table 1. Kinetic characterization of D-aspartate uptake in primary cultures of cerebellar granule cells in the absence and presence of L-*trans*-PDC and *cis*-ACBD

Inhibitor	Concn (μM)	K_m (μM)	V_{max} (nmol/min/mg)	K_i (μM)
Control	—	28 ± 3	5.1 ± 0.2	—
L-*trans*-PDC	40	57 ± 11*	4.9 ± 0.4	38
	75	77 ± 14†	4.3 ± 0.6	43
Control	—	16 ± 4	8.1 ± 0.6	—
cis-ACBD	150	34 ± 9*	8.3 ± 0.4	133
	500	86 ± 14†	8.5 ± 0.7	114

The kinetic parameters K_m and V_{max}, presented as the mean (±SD) of at least eight experimental values, were determined by weighted non-linear regression analysis using the ENZFITTER software [36]. The inhibition constants (K_i) for L-*trans*-PDC and *cis*-ACBD were calculated using the equation for linear competitive inhibition [39]. The observed differences in the two sets of control values for K_m and V_{max} reflect different batches of cells in experiments undertaken at different periods.

Statistically significant differences (Student's *t*-test) from control are indicated: *$P < 0.05$; †$P < 0.001$.

Table 2. Kinetic characterization of D-aspartate uptake in primary cultures of cortical astrocytes in the absence and presence of L-*trans*-PDC and *cis*-ACBD

Inhibitor	Concn (μM)	K_m (μM)	V_{max} (nmol/min/mg)	K_i (μM)
Control	—	64 ± 11	8.9 ± 0.6	—
L-*trans*-PDC	75	102 ± 14*	6.9 ± 0.8	98
	150	149 ± 29†	7.4 ± 0.7	112
Control	—	51 ± 8	4.5 ± 0.6	—
cis-ACBD	250	147 ± 17‡	5.1 ± 1.9	145
	700	303 ± 47‡	5.4 ± 1.7	140

The kinetic parameters K_m and V_{max}, presented as the mean (±SD) of at least eight experimental values, were determined by weighted non-linear regression analysis using the ENZFITTER software [36]. The inhibition constants (K_i) for L-*trans*-PDC and *cis*-ACBD were calculated using the equation for linear competitive inhibition [39].

Statistically significant differences (Student's *t*-test) from control are indicated: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

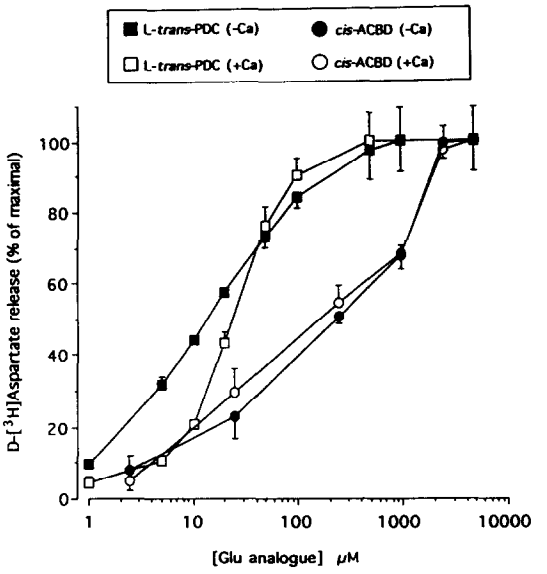


Fig. 2. Dose–response profiles for L-*trans*-PDC and *cis*-ACBD in evoking D-[^{3}H]aspartate release from cultured cerebellar granule cells. Primary cultures of granule cells were loaded with D-[^{3}H]aspartate before stimulation in a continuous superfusion system with 1 μM–5 mM L-*trans*-PDC (■, □) or *cis*-ACBD (●, ○) in the absence (■, ●) and presence (□, ○) of 1.3 mM $CaCl_2$. Data points are the mean (±SD) of four experiments and represent at least six stimulations at each drug concentration.

non-linear regression analysis of the kinetic data showed that, in both cell types, L-*trans*-PDC and *cis*-ACBD acted as linear competitive inhibitors of the carrier exhibiting a significant increase in the apparent K_m with no change in V_{max} (Tables 1 and 2). L-*trans*-PDC was more potent than *cis*-ACBD as an inhibitor of D-[^{3}H]aspartate uptake both in granule cells and astrocytes, exhibiting a K_i value (~40 μM) in granule cells that was 3-fold more potent than *cis*-ACBD ($K_i \sim 120$ μM). In astrocytes, however, L-*trans*-PDC ($K_i \sim 100$ μM) is only slightly (1.4-fold) more potent than *cis*-ACBD ($K_i \sim 140$ μM). Furthermore, L-*trans*-PDC is more potent as an inhibitor of D-[^{3}H]aspartate uptake in granule cells than in astrocytes, whereas *cis*-ACBD is essentially equi-effective in both preparations.

Dose–response relationships

In the absence of any other stimulating agent, L-*trans*-PDC and *cis*-ACBD dose-dependently evoked the release of previously accumulated D-[^{3}H]-aspartate from primary cultures of cerebellar granule cells (Fig. 2). The effect of L-*trans*-PDC and *cis*-ACBD is stimulating D-[^{3}H]aspartate release was evaluated both in the absence and presence of extracellular Ca^{2+}. The EC_{50} values for L-*trans*-PDC evoked release of D-[^{3}H]aspartate were, respectively, 19 ± 2 μM and 25 ± 6 μM in the absence and presence of Ca^{2+}. In the case of *cis*-ACBD, the EC_{50} values determined in the absence and presence of extracellular Ca^{2+} were, respectively, 220 ± 30 μM and 182 ± 28 μM. The Ca^{2+}-dependency of D-[^{3}H]aspartate release was always undertaken on the same batches of granule cells; the results obtained indicating that the maximal stimulated release (in terms of dpm) was independent of the presence of Ca^{2+}. Variations in the absolute release could however be detected between different batches of cells. For this reason, release is expressed as a per cent of maximal.

Effect of EAA receptor antagonists

The possible involvement of ionotropic EAA receptors in mediating either the L-*trans*-PDC or *cis*-ACBD evoked release of D-[^{3}H]aspartate was investigated. When granule cells were superfused with 20 μM L-*trans*-PDC or 180 μM *cis*-ACBD alone or co-administered with either the selective NMDA receptor antagonist, D-APV (300 μM), or the selective AMPA receptor antagonist, CNQX (25 μM), no differences were detected in the magnitude of the evoked release compared to that measured in the absence of antagonist (Table 3). This observation is consistent with a lack of EAA receptor involvement in mediating either L-*trans*-PDC or *cis*-ACBD evoked release of D-[^{3}H]aspartate.

Effect of Na^+ *substitution*

A plausible alternative mechanism to explain the L-*trans*-PDC or *cis*-ACBD evoked release, of D-[^{3}H]aspartate would include reversal of Na^+-dependent plasma membrane Glu transport following inward co-transport with either L-*trans*-PDC or *cis*-ACBD. This being the case, substitution of Na^+ in the extracellular medium would be expected to attenuate release. A typical profile illustrating the L-*trans*-PDC evoked release of D-[^{3}H]aspartate is presented in Fig. 3; a similar profile being observed when *cis*-ACBD was used to evoke release. It can be seen that replacement of Na^+ with an equimolar concentration of choline ions causes marked attenuation of evoked release; this effect being

Table 3. Effect of EAA receptor antagonists on L-*trans*-PDC and *cis*-ACBD stimulated release of D-[^{3}H]aspartate in primary cultures of cerebellar granule cells

Addition	Stimulated release of D-[^{3}H]aspartate (dpm above basal)		
	Control	+D-APV	+CNQX
L-*trans*-PDC	5065 ± 1137	5672 ± 933	7151 ± 396
cis-ACBD	17,742 ± 3255	18,346 ± 2390	15,930 ± 3243

Primary cultures of mouse cerebellar granule cells were loaded with D-[^{3}H]aspartate as described in Materials and Methods. Every 4 min, cells were stimulated with either 20 μM L-*trans*-PDC or 180 μM *cis*-ACBD in the absence (control) and presence of 300 μM D-APV or 25 μM CNQX. Stimulated release represents the cumulative release (dpm) per 1 min stimulation following correction for basal efflux. Results are presented as the mean (±SD) of four experiments, each comprising at least three stimulations per treatment.

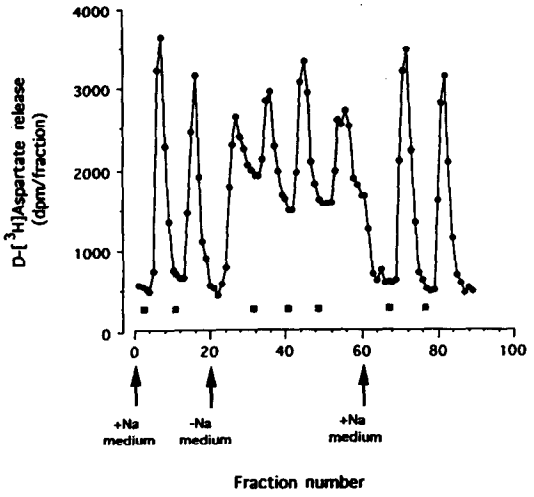


Fig. 3. Effect of sodium ion substitution on L-*trans*-PDC stimulated release of D-[^{3}H]aspartate from cultured cerebellar granule cells. Granule cells were loaded with D-[^{3}H]aspartate as described in Materials and Methods and superfused with HEPES-buffered medium (+Na medium) or with the same buffer in which Na^+ were replaced by choline ions (−Na medium). Where indicated (■) cells were stimulated for 1 min with 20 μM L-*trans*-PDC in the appropriate medium. Release is expressed as dpm in individual fractions which have not been corrected for basal efflux. The superfusion profile represents a single experiment.

reversed upon re-introduction of Na^+ into the superfusion medium. Quantitatively, 20 μM L-*trans*-PDC and 180 μM *cis*-ACBD evoked a release of D-[^{3}H]aspartate that was reduced by 60% and 89%, respectively, when Na^+ were removed from the superfusion medium. It is also readily seen from Fig. 3 that an increase in the basal release of D-[^{3}H]-aspartate occurs when the superfusion medium is depleted of Na^+; presumably, a consequence of the inverse ($[Na^+]_{in} > [Na^+]_{out}$) gradient created by removal of extracellular Na^+. The initial change-over to a Na^+-depleted superfusion medium is accompanied by an apparent peak of D-[^{3}H]aspartate release in the absence of any stimulating agent; this "overshoot" possibly reflecting the system adjusting to the altered ionic conditions.

DISCUSSION

This study shows that the conformationally restricted Glu analogues, L-*trans*-PDC and *cis*-ACBD, are competitive inhibitors of Glu transport in primary cultures of neurons and astrocytes, and moreover, that both compounds are themselves likely to be transported by the respective carriers. L-*trans*-PDC [29] and *cis*-ACBD [30] have previously been reported to function as equipotent and selective inhibitors of high-affinity Glu transport in synaptosomes. However, in the present study, L-*trans*-PDC is shown to be more potent than *cis*-ACBD as a competitive inhibitor of D-aspartate uptake both in cerebellar granule cells and astrocytes. This observation is in agreement with recent molecular modelling studies [8] which show that L-*trans*-PDC possesses superior binding characteristics to *cis*-ACBD in being accommodated at the substrate binding site of the synaptosomal Glu transporter. The potency of L-*trans*-PDC and *cis*-ACBD as inhibitors of the Glu transporter in cultured cells is however somewhat lower than that observed in synaptosomes [8, 29, 30]. Furthermore, the variation in the K_i values for L-*trans*-PDC and *cis*-ACBD with regard to neuronal versus glial (astrocyte) systems supports a considerable body of evidence obtained from biochemical/pharmacological [e.g. 40] and molecular biological [9–11] studies, suggesting the presence of Glu transporter subtypes in the CNS.

The greater potency of L-*trans*-PDC compared to *cis*-ACBD in evoking D-[^{3}H]aspartate release from granule cells correlates well with the potency of these compounds as inhibitors of Glu transport. Moreover, in release studies, since no addition stimulating agents were present in the superfusion medium, it can be concluded that L-*trans*-PDC and *cis*-ACBD display a wider-ranging action than being solely inhibitors of Glu transport. Their ability to evoke a dose-dependent, saturable release of D-[^{3}H]aspartate is consistent with both compounds

acting as EAA receptor agonists and/or substrates for the Glu transporter. Since ionotropic EAA receptor subtypes are expressed in primary cultures of cerebellar granule cells [34, 41], a Ca^{2+}-dependent, receptor-mediated mechanism of evoked-release must be considered. This mechanism can be discounted, however, on two grounds: (i) selective antagonists of the NMDA and AMPA/KA receptors fail to attenuate release evoked by either L-*trans*-PDC or *cis*-ACBD. This observation is in agreement with competitive binding studies which showed that neither L-*trans*-PDC [29] nor *cis*-ACBD [42] had any appreciable effect in displacing radiolabelled EAA agonists from any of the ionotropic receptor subclasses; (ii) the L-*trans*-PDC and *cis*-ACBD-evoked release of D-[^{3}H]aspartate from granule cells is independent of extracellular Ca^{2+}. If the action of either Glu analogue was receptor-mediated, activation of EAA receptors would result in depolarization and consequent Ca^{2+} entry which would initiate exocytotic transmitter release.

Other plausible mechanisms which may explain the action of these Glu analogues are (i) depolarization-induced carrier reversal [43] following the electrogenic uptake of EAAs via Na^+-coupled symport [44] but more probably (ii) heteroexchange of D-aspartate for either L-*trans*-PDC or *cis*-ACBD. This latter mechanism requires that both L-*trans*-PDC and *cis*-ACBD act as substrates of the Glu transporter. Their ability to act as competitive inhibitors of the transporter and to exhibit a saturability in evoking D-[^{3}H]aspartate release is consistent with this notion.

Further evidence to support the role of the transporter in mediating D-[^{3}H]aspartate release is provided by the demonstration that L-*trans*-PDC and *cis*-ACBD-evoked release is markedly attenuated by removal of Na^+ from the extracellular medium. The operation of the transporter in the uptake direction is a Na^+-dependent process; the $[Na^+]_{out} > [Na^+]_{in}$ gradient being essential to couple the "uphill" movement of Glu against an approximate 35,000-fold concentration gradient which is normally maintained between the extracellular fluid and cytosol of glutamatergic neurons [45]. Reversal of this Na^+-gradient would encourage the "downhill" movement of Glu (and aspartate) out of the neuron via the transporter. The increased basal efflux of D-[^{3}H]aspartate observed in release (superfusion) experiments when the superfusion medium is depleted of Na^+ is consistent with such a phenomenon.

The results can be incorporated into a model, depicted in Fig. 4. No account has been taken of the well-established role of K^+ or the stoichiometry of Na^+ and K^+ translocation [13, 46]. The influx cycle is started extracellularly by binding of D-[^{3}H]-aspartate to the unoccupied transporter species (T*) followed by the binding of Na^+. This ternary complex is translocated through the plasma membrane resulting in the transfer of D-[^{3}H]aspartate and Na^+ to the intracellular environment. In this way the cell is "loaded" with D-[^{3}H]aspartate. Normally, the unloaded transporter (T*) is returned to the extracellular membrane surface as a binary complex with K^+ [13, 46] in readiness for another cycle. In

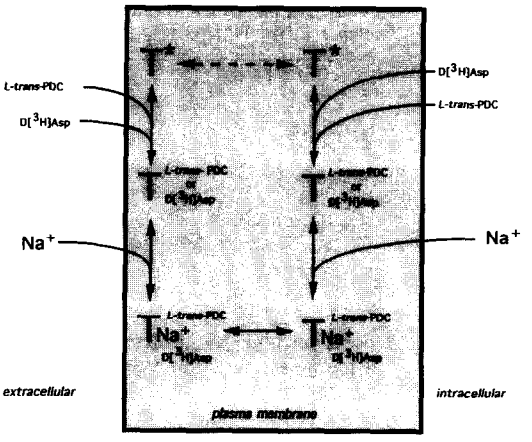


Fig. 4. Hypothetical model to explain the mechanism of L-*trans*-PDC (or *cis*-ACBD) induced release of D-[^{3}H]-aspartate. Modified from [46]; see text for explanation. When viewing the model, it should be understood that L-*trans*-PDC (or *cis*-ACBD) does not bind simultaneously with D-[^{3}H]aspartate to the transporter (T). Therefore, Na^+ can bind either to a T/L-*trans*-PDC or a T/D-[^{3}H]-aspartate species to form a ternary complex.

order for extracellular L-*trans*-PDC or *cis*-ACBD to induce release, it is most probable that a heteroexchange mechanism operates whereby D-[^{3}H]aspartate attaches to the intracellular binding site and is translocated (Fig. 4; going in a clockwise direction) to the outside and released; L-*trans*-PDC or *cis*-ACBD then binds to the outside and is translocated (Fig. 4; going in an anti-clockwise direction) into the cell where it is released and exchanged for another molecule of D-[^{3}H]aspartate. With regard to the present cell culture model, the dependence on extracellular Na^+ of L-*trans*-PDC and *cis*-ACBD induced D-[^{3}H]aspartate release could be explained on the basis of an ordered mechanism in which Na^+ binds only after either Glu analogue. However, since the removal of Na^+ does not abolish induced release totally it is possible that a random order of extracellular Na^+ binding may occur. More detailed kinetic information would be required to resolve this uncertainty.

In conclusion, this study provides strong evidence to indicate that L-*trans*-PDC and *cis*-ACBD, two potent and selective inhibitors of the high-affinity, Na^+-dependent plasma membrane Glu transporter, are described more accurately as competitive substrates. Such characteristics may apply to many other so-called Glu transport "blockers", a term that may be used mistakenly as being synonymous with "non-transportability". Moreover, it is important to recognise that, by being substrates for the transporter, these compounds are able to exchange with endogenous EAAs; the latter (unlike many inhibitors) then being able to exert extracellular effects (e.g. receptor activation). The present findings may therefore be of particular use to those who use such compounds as pharmacological tools.

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