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Steroid Hormone-Inducible Expression of the GLT-1 Subtype of High-Affinity L-Glutamate Transporter in Human Embryonic Kidney Cells

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The cDNA encoding the predominant rat brain high-affinity L-glutamate transporter GLT-1 was isolated and subcloned into the pIND expression vector for the establishment of steroid hormone inducible expression *in vitro* using the ecdysone-inducible mammalian expression system. Steroid hormone-inducible expression was demonstrable in a stable cell line designated HEK/GLT-1. Treatment of HEK/GLT-1 cells with 10 μM ponasterone A for 24 hincreased the maximum velocity (V_{max}) of Na^+-dependent L-glutamate uptake by greater than 10-fold, as compared with the uninduced cells. Equivalent levels of L-glutamate transport capacity were observed in the uninduced GLT-1 cell line and the host cell line indicating that the expression of GLT-1 was tightly regulated. To confirm that the increased L-glutamate uptake observed in HEK/GLT-1 cells following induction was attributable to the expression of GLT-1, rather than the up-regulation of the endogenously expressed EAAT3 subtype present in the host cells, we evaluated the effects of the selective GLT-1 inhibitors dihydrokainate (DHK) and kainate. Both DHK and kainate produced concentration-dependent inhibition of the L-glutamate uptake into HEK/GLT-1 cells, and the estimated IC_{50} values were consistent with those described for the cloned GLT-1. These results demonstrate that the expression of GLT-1 can be tightly regulated *in vitro* using the ecdysone system. © 1999 Academic Press

Key Words: **GLT-1; L-glutamate transporter; ecdysone; dihydrokainate; kainate.**

L-Glutamate is the predominant excitatory amino acid transmitter in the mammalian central nervous system (1). The inactivation of synaptically released L-glutamate is achieved by its reuptake into neurones and glial cells, a process which is mediated by a family of high-affinity Na^+-dependent L-glutamate transporters (2–4). In addition, these transporters are also responsible for maintaining extracellular concentrations of L-glutamate below a threshold which triggers excitotoxicity. To date, cDNAs encoding five L-glutamate transporters, GLAST/EAAT1, GLT-1/EAAT2, EAAC1/EAAT3, EAAT4 and EAAT5, have been isolated from both human and non-human species (5–10). Although five members of this transporter family have been identified it is known that the GLT-1/EAAT2 subtype accounts for the bulk of the uptake capacity which can be measured in preparations derived from adult rat cortex and therefore represents the most important subtype (11). Development of systems for the expression of GLT-1 are required for the analysis of the pharmacological and functional properties of the transporter protein. In addition, such systems will provide the means to develop assays for the identification of novel inhibitors and modulators.

Recently, the ecdysone inducible mammalian expression system was described as an appropriate system for both the *in vitro* and *in vivo* regulation of gene expression (12). The insect steroid hormone ecdysone is responsible for the activation of metamorphosis in *Drosophila melanogaster*, an effect mediated by a heterodimer of the functional ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP) which activates gene expression. It has been demonstrated that responsiveness to the synthetic ecdysone analogs muristerone A or ponasterone A can be engineered in mammalian cells by co-expression of the EcR with the mammalian homologue of the USP gene, the retinoid X receptor (RXR) (12). Using this system the expression of a chosen cDNA can be placed under the control of the EcR/RXR heterodimer which in theory activates gene expression only in the presence of exogenously supplied hormone. A number of advantages of the ecdysone system over tetracycline based strategies have been claimed including its use as a naturally evolved inducible system, the lipophilic nature of the ecdysone analogs, short half life and favourable pharmacokinetics and lack of toxicity or teratogenicity of the synthetic analogs (13).

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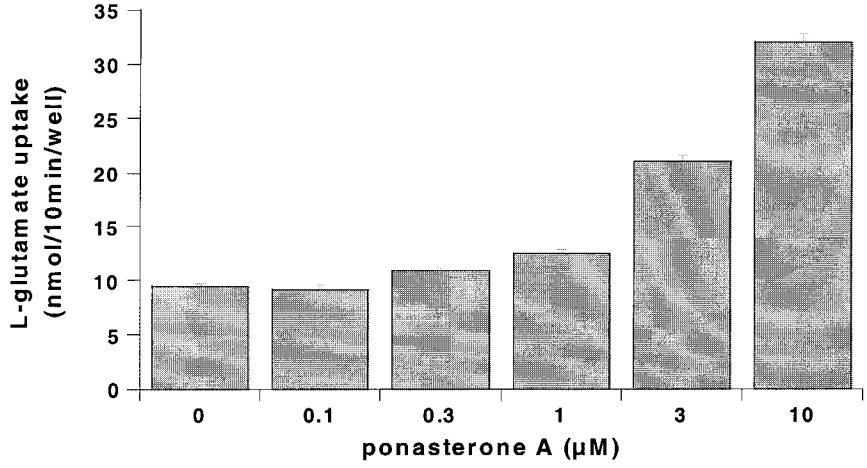

FIG. 1. Concentration dependence of the ponasterone A induction of Na^+-dependent L-glutamate transport activity in HEK/GLT-1 cells. Cells were exposed to the indicated concentrations of ponasterone A for 24 h prior to the assessment of L-glutamate uptake. Data represent mean values ± SEM (n = 3).

Relatively few studies have examined the use of the ecdysone system for the controlled expression of mammalian genes. In order to examine the feasibility of using the ecdysone system for control of gene expression of the predominant L-glutamate transporter GLT-1, we have isolated the cDNA encoding this transporter from rat brain and attempted to establish steroid hormone inducible GLT-1 expression *in vitro* using EcR 293 cells. The EcR 293 cell line is a commercially available line which has been derived from HEK 293 cells by incorporation of the regulatory vector pVgRXR which encodes both the RXR and EcR receptors. The benefit of using this cell line as a host is that it already encodes for these regulatory proteins, and the pIND vector containing the gene of interest, which is under the control of a minimal heat shock promoter and five upstream ecdysone response elements, can be transfected into these cells. In addition, HEK 293 cells exhibit only a small endogenous capacity for L-glutamate transport, which has been attributed to expression of the EAAC1/EAAT3 subtype (14), thus making them a suitable host for GLT-1 transfection provided that a good level of expression of the protein can be attained.

MATERIALS AND METHODS

Cloning of the rat GLT-1 subtype. The GLT-1 coding region was first obtained as two overlapping fragments using the following two sets of synthetic oligonucleotide primers: 5′-ATAGGATCCATGGCATCAACCGAGGGT-3′, 5′-TGCGAATTCAGGCTTGTACCAGGTTCTCC-3′, and 5′-GACGAATTCAGAACGACGAGGTGTCCAGC-3′, 5′-CGCAAGCTTCGGGTCATTATTTTTCACG-3′. These primer pairs were designed to amplify fragments 97–648 and 571–1828, respectively, of the published rat GLT-1 sequence (6). The double stranded cDNAs were synthesised directly from rat brain poly(A)$^+$ RNA (Clontech) using the Superscript One-Step RT-PCR system from GibcoBRL. The two amplified cDNA fragments were purified and cloned in pBluescript SK(+) and after sequence confirmation the full length GLT-1 cDNA was obtained by combining the two fragments with their shared unique XhoII site in pBluescript SK(+).

Generation of stable GLT-1 expressing cell line. GLT-1 was subcloned from pBluescript SK(+) into the pIND expression vector from Invitrogen prior to transfection into EcR 293 cells (Invitrogen) using the lipofectamine reagent (GibcoBRL). Several clones were isolated based on their resistance to zeocin and G418 and a preliminary evaluation of ponasterone A inducible L-glutamate uptake was used to screen the clones before selection of the clone designated HEK/GLT-1.

L-Glutamate transport assays. Uptake of L-[^{3}H]glutamate was assayed at room temperature with cells prepared in 24 well plates precoated with poly-D-lysine. Cells were exposed to medium containing ponasterone A for 24 h before assessment of uptake. All incubations were in a HEPES buffered saline (HBS) solution containing HEPES (10 mM), Tris Base (5 mM), NaCl (140 mM), KCl (2.5 mM), $CaCl_2$ (1.2 mM), $MgCl_2$ (1.2 mM), K_2HPO_4 (1.2 mM) and glucose (10 mM) at pH 7.4. Na^+-free HBS was prepared by equimolar replacement of Na^+ with choline. Immediately before assay cells were washed three times with HBS before being exposed to L-[^{3}H]glutamate substrate for 10 min. For the determination of saturable uptake cells were incubated with 1 μCi/assay L-[^{3}H]glutamate in the presence of increasing concentrations of unlabelled L-glutamate in the range 1–100 μM. In the experiments where transport inhibitors were studied 1 μM L-[^{3}H]glutamate was used and the compounds were examined over the concentration range 0.1–1000 μM. Assays were stopped after 10 min by aspiration followed by two rapid washes with ice cold Na^+-free HBS and accumulated radioactivity was measured by liquid scintillation counting after the addition of 0.5 N NaOH to each well. Protein measurements were performed using the commercially available BioRad assay kit.

Data analysis. In each experiment parallel determinations using Na^+-free HBS were performed and the results obtained were used to correct for Na^+-independent uptake. Consequently all data shown represents the Na^+-dependent L-glutamate uptake. L-glutamate uptake in the saturation analysis was calculated as pmol min^{-1} mg^{-1} protein and the Eadie-Hofstee linear transformation was used for the estimation of the kinetic parameters K_m and V_{max}. The effects of transport inhibitors were expressed as % control uptake observed in the absence of added compound, and IC_{50} values were determined by fitting the log-concentration response curves using a four parameter logistic function.

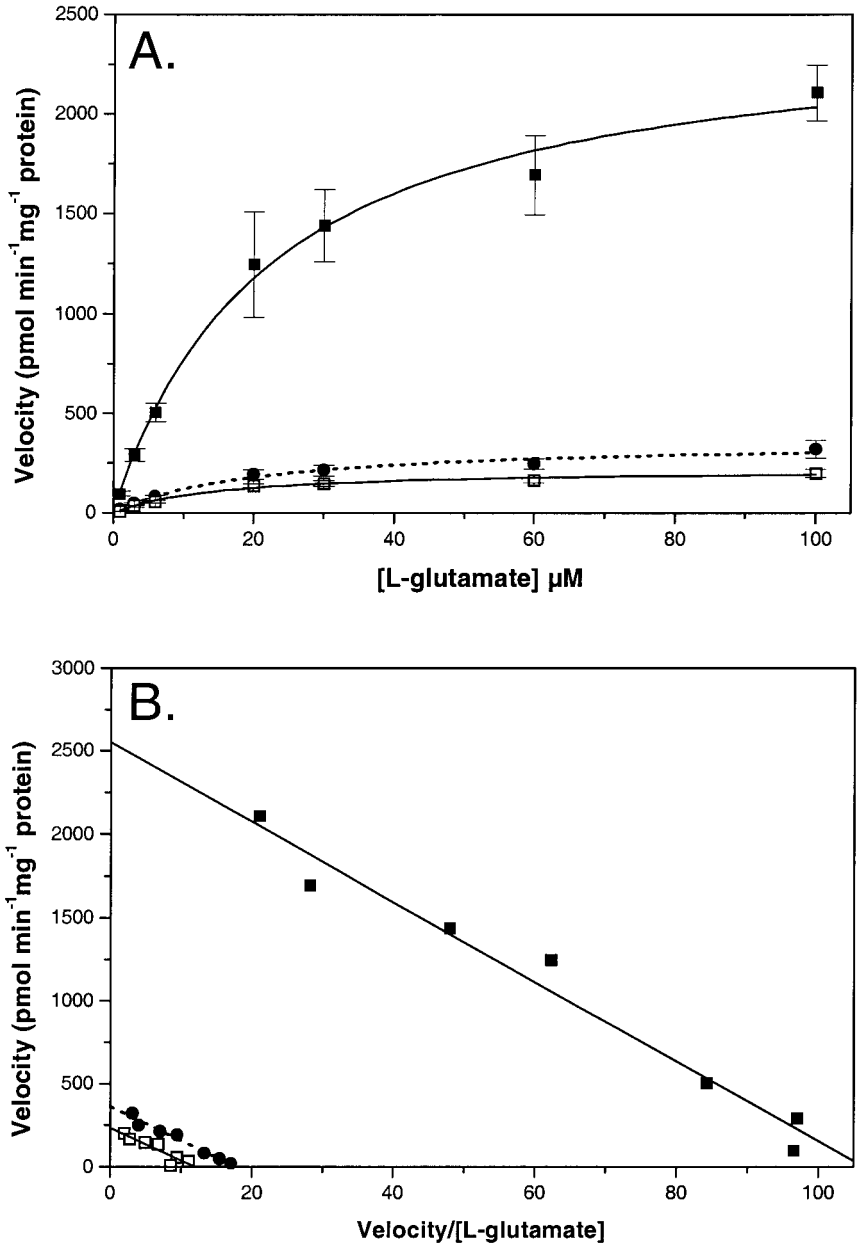

FIG. 2. Kinetic characterisation of L-glutamate uptake. (A) Saturation isotherms for Na^+-dependent L-glutamate uptake into induced HEK/GLT-1 cells (solid squares), uninduced HEK/GLT-1 cells (open squares), and EcR 293 cells (solid circles). (B) Eadie-Hofstee transformation of the saturable uptake data depicted in A. Kinetic constants estimated from the linear transformations are presented in Table 1.

RESULTS

Ponasterone A Induction of L-Glutamate Transport Activity in Cells Transfected with GLT-1

The ability of ponasterone A exposure to induce the expression of L-glutamate transport activity in HEK cells transfected with GLT-1 is illustrated in Fig. 1. HEK/GLT-1 cells were incubated in medium containing ponasterone A at the indicated concentrations for 24 h prior to the assessment of L-glutamate uptake. The Na^+-dependent uptake of L-glutamate observed in the absence of ponasterone A induction can be attributed to the expression of an endogenous L-glutamate transport activity in HEK cells. Ponasterone A was effective in the range 0.3–10 μM, inducing a concentration dependent increase in the L-glutamate uptake capacity in the HEK/GLT-1 cells. For subsequent study 10 μM was chosen as the concentration for induction and it should be noted that higher concentrations could not be examined due to the limitations of solubility with the steroid hormone. The activity of the expressed GLT-1 compared to that of the endogenously expressed

TABLE 1

Kinetic Parameters of L-Glutamate Transport

	V_{max} (pmol min^{-1} mg^{-1})	K_m (μM)
HEK/GLT-1 induced	2551	24
HEK/GLT-1 uninduced	232	19
EcR 293	360	20

transport activity is best compared by comparison of their kinetic profiles and this is presented in Fig. 2. HEK/GLT-1 cells were incubated for 24 h in the absence or presence of 10 μM ponasterone A before the analysis of saturable L-glutamate transport. As illustrated in Fig. 2A, the HEK/GLT-1 cells exhibited a greater than 10-fold higher maximum capacity for Na^+-dependent L-glutamate accumulation when compared with the uninduced cells. In addition, equivalent levels of L-glutamate uptake were observed in the uninduced HEK/GLT-1 cells and the host cells, indicating that the expression of GLT-1 was tightly regulated by the availability of exogenous inducing agent. Figure 2B shows the Eadie-Hofstee linear transformation of the saturable uptake data in Fig. 2A, and the kinetic parameters estimated from this analysis are presented in Table 1.

Pharmacological Profile of Expressed GLT-1

Although an endogenous L-glutamate uptake activity can be measured in HEK cells this activity can be shown to be insensitive to the selective GLT-1 inhibitor dihydrokainate (DHK) suggesting that another subtype mediates this activity. Recently, the identity of the endogenous L-glutamate transporter expressed in HEK cells was established (14) with the EAAT3 subtype being responsible for the activity in these cells. In order to clearly establish the expression of GLT-1 following induction we examined the effect of the selective GLT-1 inhibitors DHK and kainate on L-glutamate uptake into HEK/GLT-1 cells following 24 h ponasterone A (10 μM) induction. In addition, the effects of the non-selective L-glutamate uptake inhibitors L-CCG-III ((2S,3S,4R)-2-(carboxycyclopropyl)glycine) and *trans*-2,4-PDC (L-*trans*-pyrrolidine-2,4-dicarboxylate) were also examined. The log-concentration response curves (Fig. 3) clearly demonstrate that L-glutamate uptake into HEK/GLT-1 cells was inhibited by the two GLT-1 selective compounds, and also by the two non-selective inhibitors examined. Table 2 presents the estimated IC_{50} values generated from the log-concentration response curves for the four compounds which were evaluated, with the rank order of inhibitory activity being L-CCG-III = *trans*-2,4-PDC > DHK > kainate. The inhibitory activities observed for DHK and kainate are consistent with the majority of the uptake capacity in HEK/GLT-1 cells being attributed to the expressed GLT-1. Additionally, the observation that both DHK and kainate failed to completely block L-glutamate transport (84 and 83% inhibition at 1 mM, respectively), whilst the two non-selective inhibitors were able to completely inhibit the uptake activity in HEK/GLT-1 cells is consistent with the expression of a small endogenous capacity for transport not mediated by the GLT-1 subtype.

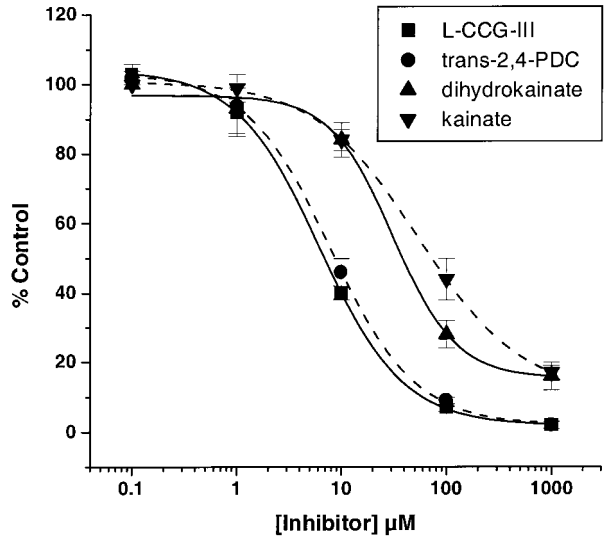

FIG. 3. Log-concentration response curves for the inhibition of L-glutamate uptake into HEK/GLT-1 cells by selective and non-selective transport inhibitors. Cells were exposed to 10 μM ponasterone A for 24 h followed by L-glutamate uptake measurements in the absence (control) and presence of inhibitors at the indicated concentrations. Data represent mean values ± SEM (n = 3) and the estimated IC_{50} values obtained from the fitted curves are presented in Table 2.

DISCUSSION

In this study we set out to examine the feasibility of using the ecdysone inducible mammalian expression system for the control of gene expression of the predominant rat brain L-glutamate transporter GLT-1 *in vitro*. Our results establish that steroid hormone inducible expression of GLT-1 can be achieved in HEK 293 cells at levels which are considerably greater than the endogenous non-GLT-1 transport activity expressed by these cells. In the presence of maximally

TABLE 2

Pharmacology of the L-Glutamate Transporter GLT-1

L-CCG-III	6.2 ± 0.2
trans-2,4-PDC	7.9 ± 0.4
Dihydrokainate	31.2 ± 8.9
Kainate	54.4 ± 4.1

Note. IC_{50} values (μM) ± SEM from 3 experiments.

stimulating concentration of steroid hormone (ponasterone A) the L-glutamate transport activity measured in the HEK/GLT-1 cell line, which can be attributed to the expressed GLT-1, is estimated to represent greater than 85% of the total activity. In terms of the absolute expression level attained in these cells (2551 pmol min^{-1} mg^{-1} protein), the transport capacity is very similar to that observed in native preparations, such as in rat cortical synaptosomes, where Vmax values in the range 1–3 nmol min^{-1} mg^{-1} protein are typically reported.

By measuring the sensitivity to the selective GLT-1 inhibitors DHK and kainate we were able to demonstrate the extent of GLT-1 expression in these cells. The estimated IC_{50} values determined in this study for these two compounds are in very good agreement with those described recently for the GLT-1 subtype expressed constitutively in either MCB or LMTK− cells (15). Values in the range 30–70 μM were estimated for DHK and kainate, for the inhibition of L-glutamate uptake into MCB or LMTK− cells mediated by GLT-1 (15), as compared with values of 31 and 54 μM, respectively, reported here. Furthermore, the inhibitory activities of both DHK and kainate on the human EAAT2 homolog have been reported in transiently transfected COS cells (8) and in permanently transfected MDCK cells (16) and the documented IC_{50} values (15–60 μM) are comparable to those described for GLT-1. Since the human EAAT2 and rat GLT-1 species homologs are highly conserved at both the nucleic acid and protein level, similar inhibitory potencies for these selective compounds is not surprising.

In the original report describing the use of the ecdysone system for controlled gene expression, success was achieved both *in vitro* using CV-1 and HEK 293 cells, and also *in vivo* with the generation of transgenic mice exhibiting muristerone responsive induction of gene expression. In the case of the GLT-1 protein this raises the intriguing question of whether it might be possible to develop transgenic animals in which the expression of GLT-1 might be increased with steroid hormone administration. Studies have demonstrated that reduction of the expression of GLT-1 in mice by either antisense knockdown (17) or gene knockout (18) produces animals which exhibit greater susceptibility to neurodegenerative insults. These observations are consistent with the predominant role of GLT-1 in the clearance of L-glutamate in the adult brain. From these observations it would be predicted that the overexpression of GLT-1 would produce animals with greater resistance to neurodegenerative insults, by virtue of their greater capacity for the clearance of excitotoxic levels of L-glutamate. The results presented in this study at least demonstrate the feasibility of using the ecdysone system for steroid hormone inducible expression of GLT-1 *in vitro*.

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