Inducible expression of the GLT-1 glutamate transporter in a CHO cell line selected for low endogenous glutamate uptake

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Received 16 December 1997

Abstract Inducible expression of the mammalian glial cell glutamate transporter GLT-1 has been established in a CHO cell line selected for low endogenous Na^+-dependent glutamate uptake by [^{3}H]aspartate suicide selection. Culturing the cells in doxycycline-containing medium, to activate GLT-1 expression via the *Tet-On* system, increased uptake of the GLT-1 substrate D-aspartate 280-fold, and increased cell size. Applying glutamate to whole-cell clamped, doxycycline-treated cells evoked a transporter-mediated current with characteristics appropriate for GLT-1. This cell line provides a useful tool for further examination of the electrical, biochemical and pharmacological properties of GLT-1, the most abundant glutamate transporter in the brain.

Key words: Glutamate; Glutamate transporter; Inducible expression; CHO cell

1. Introduction

Glutamate uptake into neurones and glial cells of the central nervous system is important for helping to terminate the postsynaptic action of neurotransmitter glutamate, and for preventing the extracellular glutamate concentration from rising to neurotoxic levels [1–6]. In addition, glutamate release by reversal of the uptake process may underlie the death of neurones during brain anoxia [7]. Five distinct sodium-dependent glutamate transporters, GLAST, GLT-1, EAAC1, EAAT4 and EAAT5, have been cloned from mammals [8–12]. GLT-1 is the most abundant transporter [13], and represents about 1% of all brain protein [14]. The properties of these transporters have mainly been investigated by expressing them heterologously in toad oocytes.

It would be useful to have mammalian cell lines expressing the different glutamate transporters, because the transporters' properties might differ in mammalian and amphibian cells. Furthermore, mammalian cells are generally suitable for whole-cell clamping, which allows monitoring of transporter activity as a membrane current [15] while controlling the intracellular contents by dialysis with the pipette solution, facilitating investigation of the ionic movements driving glutamate transport [16]. Ideally, expression of glutamate transporters would be best done in a cell line with little endogenous glutamate transport, to improve the signal to noise ratio for investigations of the expressed transporter. The GLAST transporter has been stably expressed in HEK293 cells [17], but these cells might also express endogenous glutamate transporters, and glutamate apparently still evoked a current in these cells even in the absence of extracellular sodium suggesting the presence of a glutamate-activated current component in addition to the Na^+-dependent GLAST.

We therefore attempted to produce a cell line expressing GLT-1 but no other Na^+-dependent glutamate transporters or glutamate-activated currents, taking advantage of the existence of mutant CHO cell lines lacking Na^+-dependent glutamate transport [18,19]. These cells were isolated by suicide selection with [^{3}H]aspartate: aspartate is a substrate for Na^+-dependent glutamate transporters, and uptake of tritiated aspartate, followed by frozen storage to allow time for radiation damage to accumulate, results in an increase in the proportion of cells lacking Na^+-dependent uptake.

2. Materials and methods

2.1. Choice of cell line

The CHO cell line chosen for this work was the mutant Dd-B7, which maintains low endogenous Na^+-dependent glutamate/aspartate transport for more than 70 cell doublings in long-term culture [19]. Although GLT-1 could be expressed transiently in Dd-B7 cells (Igo and Ash, unpublished), clones maintaining stable expression were not obtained. We therefore turned to inducible expression of GLT-1, in the hope that the cells would tolerate this better.

2.2. Transfection and cell selection

Dd-B7 cells were cultured in DMEM/Ham's F12 cell culture medium (Gibco 21331) supplemented with 2.5 mM L-glutamine, non-essential amino acids (Gibco 11140-035, diluted 1:200), 5% newborn calf serum and 5% horse serum. The cells were kept at 37°C in a humidified incubator with 5% CO_2. Before the cells grew to confluence, they were co-transfected using Transfectam (Promega) with the pTet-On regulator plasmid (Clontech) and with rat GLT-1 cDNA (directionally inserted in the pTet response element plasmid using the *Eco*RI and *Xba*I cloning sites [20,21]). The GLT-1 insert included the 1.72 kbp coding sequence as well as 0.1 kbp of 5′ and 2.0 kbp of 3′ non-coding sequence.

Cells were grown for 14 h, replated at low density, and then grown for another 24 h, before selecting successfully transfected cells: pTet-On contains a neomycin resistance gene, allowing selection of transfected cells with the neomycin analogue geneticin (0.4 mg/ml). Ten to twelve days after starting culture in the geneticin-containing medium, geneticin-resistant clones were transferred to separate wells. From these resulting clonal cell lines, a radiotracing screen with D-[^{3}H]aspartate was used to select the cell line with the highest D-aspartate uptake induced by doxycycline (2 μg/ml), which should bind to the reverse Tet-responsive transcriptional activator (rtTA, encoded by the pTet-On regulator plasmid) allowing rtTA to activate expression of GLT-1 [20]. To do this, each clone was split, with an equal number

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PII S0014-5793(98)00036-2

of cells plated into two 1.9 cm^2 wells, allowing the background level of aspartate uptake and the uptake induced by doxycycline to be measured separately. Uptake was measured using external solution containing (mM) NaCl 137, K_2HPO_4 0.7, HEPES 10, $MgCl_2$ 1, $CaCl_2$ 1, pH 7.4. The cells were washed with 2×1 ml of this solution, then incubated (at 26°C) in 0.4 ml of this solution containing 0.2 μM D-[^{3}H]aspartate for 5 min, and finally washed with 3×1 ml of the external solution, before lysing with 1% SDS and scintillation-counting the accumulated radioactivity. Estimates of substrate influx based on whole-cell clamp measurements of the uptake current per cell suggested that the intracellular D-aspartate concentrate would increase by <100 μM over the 5 min incubation period. The selected cell line was then subcloned one more time to ensure it was clonal, and selection pressure was maintained subsequently using 0.2 mg/ml geneticin.

2.3. Whole-cell clamping

Cells were detached from their culture wells by washing with a solution containing (mM) NaCl 137, K_2HPO_4 0.7, HEPES 10, Na_2EDTA 0.5, pH 7.4, and replated onto a glass bottomed chamber which had been half-filled with external solution (see below, the divalent ions in this solution allow the cells to adhere to the chamber) on a fixed stage microscope. Cells were clamped with electrodes filled with solution containing (mM) KCl 140, $CaCl_2$ 0.5, Na_2EGTA 5, HEPES 10, $MgCl_2$ 2, Na_2ATP 1, pH 7.0, in external solution containing (mM) NaCl 140, KCl 2.5, HEPES 10, $MgCl_2$ 2, $CaCl_2$ 2.5, Na_2HPO_4 1, glucose 10, pH 7.4. Electrode resistance was approximately 5 MΩ when immersed in the external solution, 2 GΩ in cell-attached mode and the series resistance in whole-cell mode was around 10 MΩ. Series resistance voltage errors were negligible (<2 mV). The cells are approximately spherical after replating as described above, so voltage non-uniformity in the cell is also negligible. The electrode junction potential was corrected for. To correlate transporter current with the membrane area of the cell studied, cell diameter was measured from the image of the cell on a TV screen linked to a camera on the microscope.

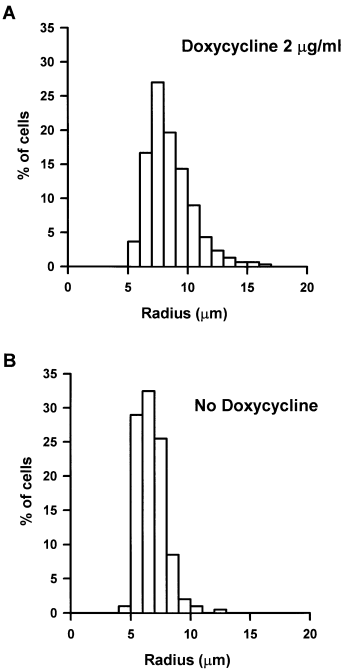

Fig. 2. Histograms showing the frequency distribution of radii of cells treated (A) or not treated (B) with 2 μg/ml doxycycline for 48 h. Doxycycline-treated cells had a mean radius of 8.52 ± 0.11 μm (mean ± S.E.M., $n = 300$), while non-treated cells had a mean radius of 6.75 ± 0.08 μm ($n = 200$), i.e. different with $P < 10^{-27}$ on Student's 2-tailed t-test.

3. Results

Activity of glutamate transporters was initially assayed by comparing the uptake of D-[^{3}H]aspartate (0.2 μM in the extracellular solution) in cells cultured in doxycycline with that in cells cultured for the same amount of time in the absence of doxycycline. Uptake was low in the absence of doxycycline (Fig. 1, open symbols), and may reflect transport by mechanisms other than Na^+-dependent transporters. With doxycycline added to the culture medium, however, D-aspartate uptake was dramatically increased within 24 h of adding the doxycycline, and reached a maximum after 36–48 h (Fig. 1). The greatest increase of uptake was produced by 2 μg/ml doxycycline, with a similar but slightly smaller increase produced by 6 μg/ml doxycycline, and about 25% less increase with 0.6 μg/ml doxycycline. For the cell batch studied in Fig. 1, the maximum upregulation of uptake (normalised by cell protein) produced by doxycycline was 280-fold. In two other batches of cells from the same selected line similar large increases were seen, by factors (not normalised by cell protein) of 117 at 24 h in one batch, and of 269 at 48 h in the other batch, in 2 μg/ml doxycycline.

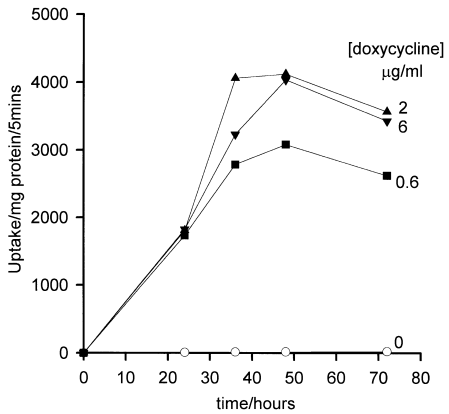

Fig. 1. Induction of glutamate/aspartate transporter expression by doxycycline. Data points show uptake of radioactive D-aspartate (arbitrary units, normalised by protein content in parallel wells of cells) in cells treated (filled symbols) or not treated (open symbols) with doxycycline. Doxycycline was added at time zero to the treated cells. The different concentrations of doxycycline used are shown by each curve.

A feature of the cells treated with doxycycline was that they appeared larger than those not treated with doxycycline. This was noticeable when the cells were growing in culture wells, and was quantified after replating the cells (see Section 2) to convert them to a conveniently measurable spherical shape (Fig. 2): their mean radius increased from 6.8 to 8.5 μm (see Fig. 2 legend), corresponding to a doubling of volume. Although this could be a non-specific effect of doxycycline, it is plausible that this volume increase was promoted by the upregulation of glutamate transport, as discussed below.

Previous work has shown that the Na^+-dependent glutamate transporters are electrogenic, so that transporter activity can be monitored as a membrane current [15]. To examine the doxycycline-induced transporter activity at a single cell level, we whole-cell clamped cells which had been treated with 2 μg/ml doxycycline for 30–48 h. Glutamate evoked an inward

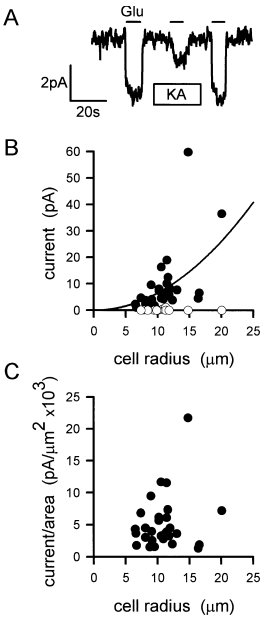

Fig. 3. Whole-cell clamp monitoring of GLT-1 expression. A: Inward current evoked by 50 μM glutamate in a cell clamped to −60 mV, and the response to the same glutamate concentration in the presence of 200 μM kainate. Kainate alone evoked no current. B: Uptake current evoked by a saturating dose of glutamate (200 or 300 μM) at −60 mV, as a function of cell radius, in cells treated (filled circles) or not treated (open circles) with 2 or 6 μg/ml doxycycline for at least 30 h. Smooth curve has the form $I = k4\pi r^2$, as predicted if the density of transporters in the cell membrane is independent of radius, with $k = 0.0052$ pA/(μm)^2, the mean value of $I/4\pi r^2$ in all the cells. C: Uptake current/cell area ($I/4\pi r^2$) from B plotted as a function of cell radius.

current (Fig. 3A) that was reduced by kainate, which is a blocker specific for GLT-1 and its human homologue EAAT2 [9,22]. The response to 50 μM glutamate was reduced by 63 ± 3% (mean ± S.E.M., 4 cells) by 200 μM kainate. With no sodium in the external solution (replaced by choline) glutamate evoked no current (6 cells, data not shown), showing both that the transporter is Na^+-dependent and that there are no other glutamate-gated currents in these cells. A detailed electrophysiological analysis of the properties of GLT-1 will be published elsewhere.

We gained the impression that the cells showing the largest glutamate uptake currents were the largest diameter cells. To determine whether this was simply because larger cells have more surface area, we plotted the current evoked by a saturating dose of glutamate as a function of the measured radius of the cell (Fig. 3B), and also plotted current per area of the cell as a function of radius (Fig. 3C). The current was too small to be reliably detected for cells below 6 μm in radius. Above this size, the current increased with radius, but the current per membrane area was roughly constant within the scatter of the data (Fig. 3C). The smooth line through Fig. 3B increases with the square of the radius, as predicted if current is proportional to area (best-fitting a curve proportional to radius^n gave $n = 2.4 \pm 0.6$). Cells not treated with doxycycline rarely showed any current change in response to glutamate (open symbols in Fig. 3B), no matter what their radius, and in the two cells which appeared to show a small response it was at the limit of detection.

4. Discussion

The results reported here establish the feasibility of controlling glutamate transporter expression in mammalian cells using the *Tet-On* system. The cell line we describe should prove useful for further investigation of the properties of GLT-1, and the strategy used in its construction may be useful for expressing other transporters. By combining suicide selection of the parent CHO cell line, using radioactive substrate to select for mutants with low endogenous transport [18,19], with inducible expression of the transporter under study, we have constructed a cell line with very little background glutamate transport in which the effects of transporter expression can be studied by comparing cells treated and not treated with doxycycline.

A notable effect of treating cells with doxycycline was that their size was increased (Fig. 2). A plausible explanation of this is that doxycycline-evoked upregulation of GLT-1 accelerates uptake of glutamate and aspartate from the culture medium (which contains 50 μM glutamate and 100 μM aspartate), which would provide amino acids for protein synthesis, feed into the citric acid cycle, and also help to establish the high intracellular glutamate concentration which is needed to drive uptake of cystine, a precursor of the antioxidant glutathione, by the glutamate/cystine exchanger [19,23]. It is also possible that expression of GLT-1 increases the osmotic load on the cells, leading to swelling, or retards cell cycle progression, allowing cells to grow larger before mitosis.

We observed, in whole-cell clamp experiments, that there is more glutamate transport in larger cells (Fig. 3), but a similar density per area of the cell membrane. Coordinated cellular control of transporter expression might be expected in cells expressing transporters normally, but the random incorporation of the GLT-1 cDNA into the genome of our host cell line may preclude normal control of its expression by upstream promoters. At present it remains uncertain, therefore, whether there is an internal cellular mechanism which automatically triggers more transporter expression as cells grow larger, or whether the causality is the other way round, with cells which happen to express more glutamate/aspartate uptake being able to grow larger.

Acknowledgements: Supported by the European Community (CT 95-871), the Wellcome Trust, the Langfeldts fond, the Norwegian Research Council, and the NSF.

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