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Stimulation of EAAC1 in C6 glioma cells by store-operated calcium influx

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ABSTRACT

This study investigated how modulation of intracellular calcium alters the functional activity of the EAAC1 glutamate transporter in C6 glioma cells. Pre-incubation of C6 glioma cells with the endoplasmic reticulum Ca^{2+} ATP pump inhibitor, thapsigargin (10 μM) produced a time-dependent increase in the V_{max} for D-[^{3}H] aspartate transport that reached a maximum at 15 min (143% of control; $P<0.001$) that was accompanied by increased plasma membrane expression of EAAC1 and was blocked by inhibition of protein kinase C. Pre-incubation of C6 glioma cells with phorbol myristate-3-acetate (100 nM for 20 min) also caused a significant increase in the V_{max} of sodium-dependent D-[^{3}H]aspartate transport (190% of control; $P<0.01$). In contrast, in the absence of extracellular calcium, thapsigargin caused a significant inhibition in D-[^{3}H] aspartate transport that was not mediated by protein kinase C. Blockade of store-operated calcium channels with 2-aminoethoxydiphenyl borate (50 μM) or SKF 96365 (10 μM) caused a net inhibition of D-[^{3}H]aspartate uptake. Co-incubation of C6 glioma cells with both thapsigargin and 2-aminoethoxydiphenyl borate (but not SKF 96365) prevented the increase in D-[^{3}H]aspartate transport that was observed in the presence of thapsigargin alone. Furthermore, 2-aminoethoxydiphenyl borate, but not SKF 96365, reduced the increase in intracellular calcium that occurred following pre-incubation of the cells with thapsigargin. It is concluded that, in C6 glioma cells, stimulation of EAAC1-mediated glutamate transport by thapsigargin is dependent on entry of calcium via the NSCC-1 subtype of store operated calcium channel and is mediated by protein kinase C. In contrast, in the absence of store operated calcium entry, thapsigargin inhibits transport.

1. Introduction

Five members of the high affinity glutamate transporter family have been identified in the mammalian brain (for a review, see [1]). It is generally agreed that three subtypes, namely GLAST (EAAT1 in the human), GLT1 (EAAT2) and EAAC1 (EAAT3), together account for almost all of the glutamate taken up into both pre-synaptic neurons and glial cells (astrocytes). GLAST and GLT1 are principally astrocytic carriers, whereas EAAC1 is generally associated with neurons [2–6]. The majority of synaptically released glutamate is cleared by GLT1- and GLAST-mediated uptake into astrocytes, thereby preventing death of neurones caused by hyperactivation of glutamate receptors ('excitotoxicity'). Paradoxically, the C6 glioma cell line, which is derived from a rat astrocytoma, expresses only the EAAC1 transporter subtype [7]. It has been proposed that insufficient uptake of glutamate in glioma cells may contribute to the progression of brain tumours, because of damage to adjacent neurones through excitotoxic mechanisms initiated by elevated extracellular glutamate [8].

Previous studies on the regulation of the EAAC1 transporter by protein kinase C (PKC) have highlighted differences between the response generated in C6 glioma cells [7,9,10] and in other experimental models, including primary neuronal cultures [11] and stable cell lines over-expressing the protein (HEK_{EAAC1} cells) [12]. Modulation of PKC does not influence EAAC1-mediated transport of L-[^{3}H] glutamate in neurones [11,13] or in HEK_{EAAC1} cells [12], whereas in C6 glioma cells, the PKC activator, phorbol myristate-3-acetate (PMA) increases both the transport of L-[^{3}H]glutamate and the cell surface expression of EAAC1 [9,14,15]. In contrast, the PI3K inhibitor, wortmannin, invariably decreases glutamate transport in a range of experimental systems, including C6 glioma cells, *Xenopus* oocytes over-expressing EAAC1, neurone-enriched primary cultures, primary cerebellar neurones and Bergmann glial cells [9,13,16,17]. PI3K is also implicated in the stimulation of glutamate transport in primary cerebellar neurones by minocycline [18].

These observations have led us to question whether it is specifically calcium-dependent signalling pathways that are most variable when comparing the modulation of glutamate transport in C6 glioma cells to other experimental systems. There is currently no data on how changes in cytosolic calcium specifically influence EAAC1-mediated glutamate transport in C6 glioma cells. However, it is known that endothelin, which activates two types of calcium-permeable non-selective cation channel (NSCC-1 and NSCC-2) in C6 glioma cells, causes an increase in the uptake of glutamate and cell-

Abbreviations: 2-APB, 2-aminoethoxy diphenylborate; CaMKII, calcium-calmodulin dependent kinase II; EAAC1, excitatory amino acid carrier 1; ER, endoplasmic reticulum; NSCC, non-selective cation channel; PI3K, phosphatidylinositol-3-kinase; PMA, phorbol myristate-3-acetate; SOCC, store-operated calcium channel

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surface expression of EAAC1 following activation of endothelin-1 type A receptors [19,20].

We have measured the effect of calcium modifying agents on D-[^{3}H]aspartate transport and calcium fluxes in C6 glioma cells in an effort to identify the origin of the responsiveness of EAAC1 to calcium-dependent kinases. As predicted, we find that the response of C6 glioma cells to changes in intracellular calcium are at variance to the results that have been reported for neuronal cells expressing the EAAC1 transporter [11]. Depletion of endoplasmic reticulum (ER) stores of calcium by the Ca^{2+} ATP pump inhibitor, thapsigargin, causes a transient increase in D-[^{3}H]aspartate transport in C6 glioma cells that is dependent on store-operated calcium entry from the cytosol. Furthermore, we provide evidence that, in the absence of extracellular calcium, thapsigargin produces a net inhibition of D-[^{3}H]aspartate transport.

2. Materials and methods

2.1. Cell culture

C6 glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin/streptomycin and L-glutamine (2 mM) on 100 mm diameter cell culture Petri dishes at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Cells were gown as a monolayer and routinely passaged twice weekly.

2.2. Measurement of D-[^{3}H]aspartate transport in C6 glioma cells

Measurements of the rate of D-[^{3}H]aspartate uptake into C6 glioma cells were performed in 12-well plates. The cells were seeded at a density of 0.25×10^6 cells into each well and, on reaching 80% confluency (typically 2–3 days), were used in the uptake assays. The growth medium was removed by suction and the cells were incubated with 450 µl Krebs' bicarbonate medium (contents in mM: NaCl 109.6, KCl 4.72, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5 pH 7.4, gassed in 95% O_2/5% CO_2). The transport assay was initiated by the addition of 50 µl D-[^{3}H]aspartate solution (final concentration 10–500 µM; specific activity 296–14,800 Bq/nmol, unless otherwise stated). Time-course experiments established that transport of D-[^{3}H]aspartate was linear with time from 0–20 min (results not shown). Accordingly, transport of D-[^{3}H]aspartate into C6 glioma cells was determined for an incubation period of 5 min at 37 °C. Thereafter, the medium was removed by suction and the cell layer was washed twice with 500 µl Krebs' bicarbonate medium. The cells were then solubilised overnight in 500 µl 0.5 M NaOH. An aliquot of 300 µl of the solubilised cells was added to 5 ml scintillation cocktail (Ecoscint A) in a scintillation vial and the level of radioactivity incorporated into the cells was determined by liquid scintillation spectroscopy. The remaining 200 µl aliquot of solubilised cells was retained for protein determination by the method of Bradford et al. [21]. The sodium dependency of D-[^{3}H]aspartate transport was assessed by using sodium-free Krebs' medium (choline chloride substituted for NaCl and 8.1 mM Tris used in place of $NaHCO_3$ to bring the pH to 7.4). The rate of sodium-dependent transport was calculated by subtraction of the rate of sodium-independent transport from the rate of total transport observed in the presence of sodium. All data are represented as the sodium-dependent transport of D-[^{3}H]aspartate, unless otherwise stated. Calcium-free Krebs' medium contained EGTA (0.98 mM) in place of calcium. Calcium-modifying agents and kinase inhibitors/activators were typically added to the cell incubation medium for 15 min prior to the initiation of a D-[^{3}H]aspartate transport assay, unless otherwise stated. Preliminary experiments established that each agent had no effect on sodium-independent transport of D-[^{3}H]aspartate and that the vehicle (1% DMSO) did not affect the sodium-dependent uptake of D-[^{3}H]aspartate in the absence of test compound (results not shown).

2.3. Ratiometric measurement of intracellular [Ca^{2+}] in C6 glioma cells by confocal microscopy

Cells were seeded into 35 mm glass bottom culture dishes at a density of 0.25×10^6 cells per dish in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin/streptomycin and L-glutamine (2 mM). They were grown at 37 °C in a humidified atmosphere of 5% CO_2, 95% air until 60–75% confluent. Intracellular calcium levels were measured using two visible wavelength calcium sensitive dyes, Fluo 3 and Fura Red. Fluo 3 exhibits an increase in green fluorescence upon binding to calcium, whereas Fura Red exhibits a decrease in red fluorescence upon binding to calcium. The Fluo 3/Fura Red ratio is a good indicator of intracellular calcium levels [22]. Initially, cells were loaded with the calcium sensitive dyes by incubation with 3 µM Fluo 3 and 3 µM Fura Red acetoxymethyl esters for 1 h in Ca^{2+}/Mg^{2+}PBS buffer at 37 °C. The dye was removed, and the culture washed with Ca^{2+}/Mg^{2+}PBS buffer. Subsequently, the cultures were washed three times with buffer. Fluo 3 and Fura Red were excited at 488 nm, and fluorescence emissions at 525 nm and 660 nm were recorded simultaneously using a Carl Zeiss LSM 510 UVMETA confocal microscope. The culture was scanned for 30 s to determine a base line prior to exposure to calcium modifying agents.

Frames of 256×256 pixels were taken and the pinhole diameter was kept constant. The ratio of the fluorescence of the two dyes was calculated according to the equation: R=emission 505–535 nm (Fluo-3)/emission >560 nm (Fura Red). Plots were constructed showing fluorescence ratio on the ordinate against time on the abscissa. For each treatment, 15 cells were analysed in three independent determinations.

2.4. Measurement of cell-surface expression of EAAC1 by biotinylation assay

EAAC1 cell surface expression was measured as previously described [9] with minor modifications. C6 glioma cells were seeded in 10 cm dishes and allowed to reach 80% confluency. Plates were then rinsed twice with ice-cold PBS containing 0.1 mM Ca^{2+} and 1.0 mM Mg^{2+} (PBS Ca^{2+}/Mg^{2+}) followed by incubation in 2 ml biotin solution (sulfo-NHS-biotin, 1 mg/ml in PBS Ca^{2+}/Mg^{2+}) for 30 min at 4 °C with gentle agitation. After two rinses with PBS Ca^{2+}/Mg^{2+} containing 100 mM glycine, plates were incubated in 100 mM glycine for 30 min at 4 °C to quench unreacted biotin. Cells were lysed in RIPA buffer (100 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate containing protease inhibitors) for 1 h at 4 °C. After scraping, lysates were cleared of cell debris by centrifugation at 130,000 rcf for 20 min. An aliquot of cell lysate was saved and mixed with an equal volume of 4× Laemmli buffer (240 mM Tris–HCl, pH 6.8, 8% SDS, 4% 2-mercaptoethanol) for further analysis as the "total protein" fraction. A second aliquot of cell lysate was incubated with an equal volume of avidin-conjugated beads. After overnight incubation at 4 °C, supernatant containing non-biotinylated proteins ("intracellular protein" fraction) was recovered by centrifugation at 130,000 rcf for 15 min and mixed with an equivalent volume of 4× Laemmli buffer. Beads containing the biotinylated ("cell surface protein") fraction were washed once with RIPA buffer containing protease inhibitors, twice with a high salt buffer (50 mM Tris, 5 mM EDTA, 500 mM NaCl, 0.1% Triton X-100, pH 7.5), and twice with a no salt buffer (50 mM Tris, pH 7.5). Finally, beads were incubated with 2× Laemmli buffer (120 mM Tris–HCl, pH 6.8, 4% SDS, 2% 2-mercaptoethanol) for 30 min at 37 °C and then centrifuged at 130,000 rcf for 15 min. The supernatant containing biotinylated proteins was saved for further analysis. Fractions were stored at −20 °C. The amount of

protein in the lysate was determined using a Bio-Rad protein assay kit using the Bradford method. For Western blot analysis, approximately 20 μg protein was loaded on 10% gels, and transferred onto a nitrocellulose membrane. Blots were incubated in anti-EAAC1 (1:500) or anti-actin (1:1000), and visualised with chemiluminescence.

2.5. Data analysis

Transport experiments were conducted under conditions of initial velocity and the rate curves were calculated by non-linear regression analysis by applying the Michaelis Menten equation to the data using the Kaleidagraph® Application from Synergy Software. Kinetic data were not corrected to allow for diffusion. The results in each figure are expressed as the mean±S.E.M. of the number of observations indicated. Data were analysed by Student's unpaired *t*-test (*). Where more than two comparisons were being made, the Tukey analysis of variance (δ) was used. In each case, the difference between means was considered significant at P values of less than 0.05.

2.6. Materials

SKF 96365, thapsigargin, anti-rabbit secondary antibody and all RIPA buffer components, unless indicated otherwise, were purchased from Sigma-Aldrich, Dorset, UK; protease inhibitors were purchased from Roche, Dublin, Ireland. 2-APB was from Tocris, Avon, UK; PMA, Bis-X, H-89, KN-93, wortmannin and genistein were all supplied by Merck Biosciences, U.K. Fura Red (AM) and Fluo-3 were obtained from Invitrogen/Molecular Probes, Eugene, OR, U.S.A. and were dissolved in DMSO (3%, giving a final concentration of DMSO in each assay of 0.06%) prior to use. Rabbit anti-rat EAAC1 primary antiserum was obtained from Alpha Diagnostic International, TX, USA. Sulfo-NHS-biotin and UltraLink Immobilized Monomeric Avidin beads were obtained from Pierce (Rockford, IL, USA). 35 mm glass bottom culture dishes were obtained from MatTek Corporation, MA, U.S.A.

3. Results

3.1. Thapsigargin stimulates store-operated entry of calcium into C6 glioma cells

Following loading with Fura Red and Fluo-3, C6 glioma cells were incubated in the presence of 10 μM thapsigargin under the following conditions: thapsigargin alone, thapsigargin and zero calcium, thapsigargin and 10 μM SKF 96365, thapsigargin and 50 μM 2-APB (Fig. 1A–D). A large increase in intracellular calcium (as indicated by an increase in Fluo-3 λ_{em}/Fura Red λ_{em}) was observed following incubation of the cells with thapsigargin, which was abolished by either removal of extracellular calcium or co-incubation of the cells with 2-APB. In contrast, co-incubation of the cells with thapsigargin and SKF96363 showed a smaller increase in calcium (Fig. 1C).

3.2. Pre-incubation of C6 glioma cells with thapsigargin stimulates sodium-dependent D-[^{3}H]aspartate transport

Pre-incubation of cells with 10 μM thapsigargin for 0–40 min prior to initiation of a transport assay showed a significant increase in the sodium-dependent uptake of 50 μM D-[^{3}H]aspartate, which reached a peak at 15 min, but had returned to control values by 30 min (Fig. 2). Sodium-independent uptake of D-[^{3}H]aspartate was not affected by pre-incubation of C6 glioma cells in the presence of 10 μM thapsigargin at any of the time points tested (results not shown). Kinetic analysis of sodium-dependent D-[^{3}H]aspartate uptake by C6 glioma cells following a 15-min pre-incubation with 10 μM thapsigargin showed a significant increase (185% of control; $P<0.001$) in the maximum rate (V_{max}) of transport from 0.592±0.066 nmol/mg protein/min in cells incubated in the absence of thapsigargin to 1.10±0.02 nmol/mg protein/min in its presence (Fig. 3). No significant change in the K_M was observed (36±15 μM in control, compared to 53±3 μM following pre-incubation of the cells with 10 μM thapsigargin). Cell surface expression of EAAC1 increased

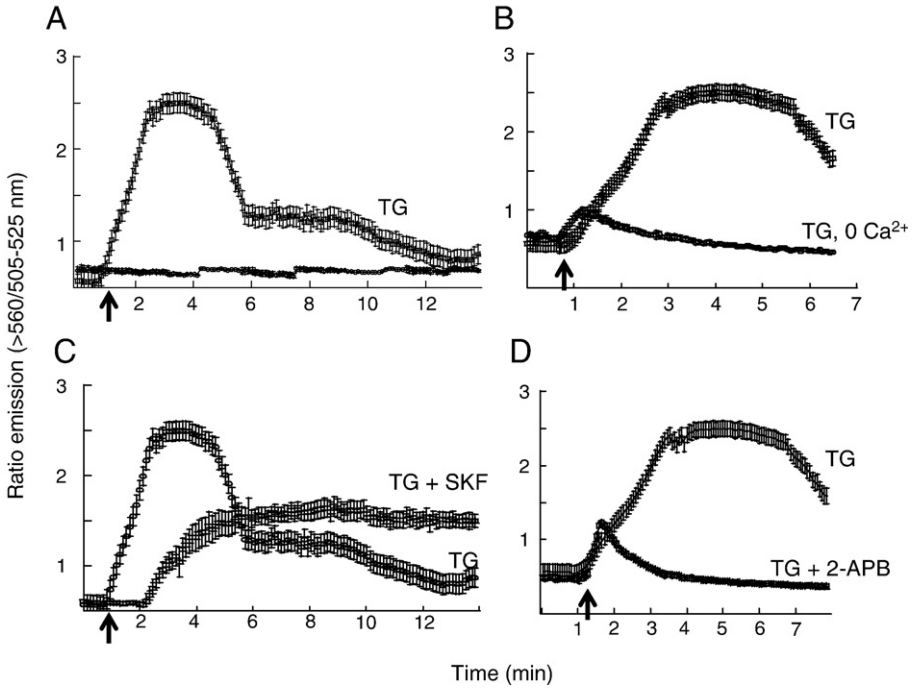

Fig. 1. Calcium signals evoked by thapsigargin in C6 glioma cells. Cells were pre-loaded with 3 μM Fura Red AM and 3 μM Fluo-3 and were incubated with 10 μM thapsigargin under the following conditions: (A) thapsigargin alone; (B) thapsigargin in the presence or absence of calcium; (C) thapsigargin in the presence or absence of 100 μM SKF 96365; (D) thapsigargin in the presence or absence of 50 μM 2-APB. Fluorescence measurements were made for 0–10 min following addition of each compound. The plots shown are the mean± SEM of 41–56 single cell traces from 3 separate experiments. TG, thapsigargin; SKF, SKF 96365.

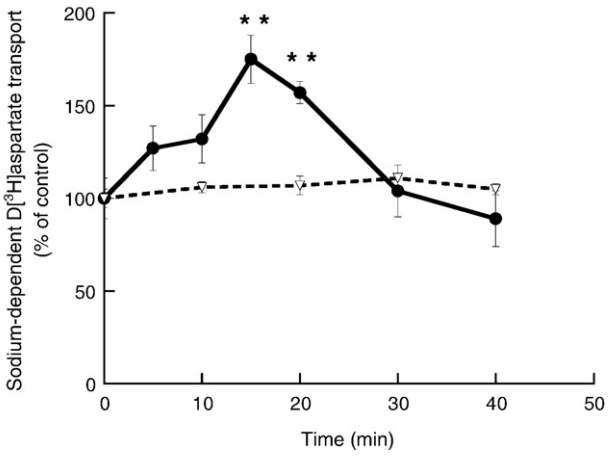

Fig. 2. Time course of the pre-incubation of C6 glioma cells with thapsigargin. C6 glioma cells were pre-incubated in the presence (○) or absence (●) of 10 μM thapsigargin for 0–40 min prior to measurement of sodium-dependent transport of 50 μM D-[^{3}H]aspartate for 5 min at 37 °C. The results are expressed as the % of transport occurring in the absence of thapsigargin and are the mean ± SEM of at least 3 separate determinations, each measured in triplicate. * $P<0.01$, compared to transport in the absence of thapsigargin.

following pre-incubation of the cells with 10 μM thapsigargin, as determined by densitometric analysis of Western blots of biotin-labelled protein.

3.3. Stimulation of D-[^{3}H]aspartate transport in C6 glioma cells by phorbol myristate-3-acetate

Pre-incubation of cells with 100 nM phorbol myristate-3-acetate (PMA) for 15 min prior to measurement of sodium-dependent D-[^{3}H]aspartate uptake led to a significant increase in the V_{max} of transport from 0.642 ± 0.052 nmol/mg protein/min in controls to 1.22 ± 0.09 nmol/mg protein/min in the presence of PMA (190% of control; $P<0.01$; Fig. 4). There was no change in the K_M observed in the presence of PMA (56 ± 12 μM) compared to its absence (43 ± 12 μM).

3.4. Removal of extracellular calcium abolishes the stimulation of D-[^{3}H] aspartate transport in C6 glioma cells by thapsigargin, but not phorbol myristate-3-acetate

Cells were pre-incubated with thapsigargin (10 μM) in calcium-free Krebs' bicarbonate medium for 30 min prior to measurement of the uptake of 1–300 μM D-[^{3}H]aspartate. In separate experiments, cells were pre-incubated with 100 nM PMA for 30 min prior to measurement of the uptake of 50 μM D-[^{3}H]aspartate. In both sets of experiments, control cells were pre-incubated in calcium-free Krebs' bicarbonate medium for 30 min before initiation of the transport assay.

In the absence of extracellular calcium, thapsigargin caused a further reduction in the rate of transport that was observed in the absence of calcium alone. Removal of extracellular calcium caused a reduction in the V_{max} of D-[^{3}H]aspartate transport from 0.512 ± 0.036 nmol/mg protein/min in control cells to 0.345 ± 0.017 nmol/mg protein/min without calcium (67% of control; $P<0.05$). Pre-incubation of the cells with thapsigargin caused a further drop in the V_{max} of transport to 0.235 ± 0.010 nmol/mg protein/min (46% of control; $P<0.01$; Fig. 5A). There was no significant difference in the K_M values of D-[^{3}H]aspartate transport under these conditions: 41 ± 10 μM (control), compared to 66 ± 9 μM (calcium-free) and 66 ± 5 μM (calcium-free with thapsigargin). The stimulation in 50 μM D-[^{3}H] aspartate uptake by PMA persisted in the absence of extracellular calcium (Fig. 5B).

3.5. Inhibition of protein kinase C blocks the stimulation of D-[^{3}H] aspartate transport by thapsigargin in C6 glioma cells

Pre-incubation of cells in the presence of both thapsigargin (10 μM) and the protein kinase C inhibitor, bisindolylmaleimide (Bis-X; 1 μM) for 15 min prior to measurement of D-[^{3}H]aspartate (50 μM) transport

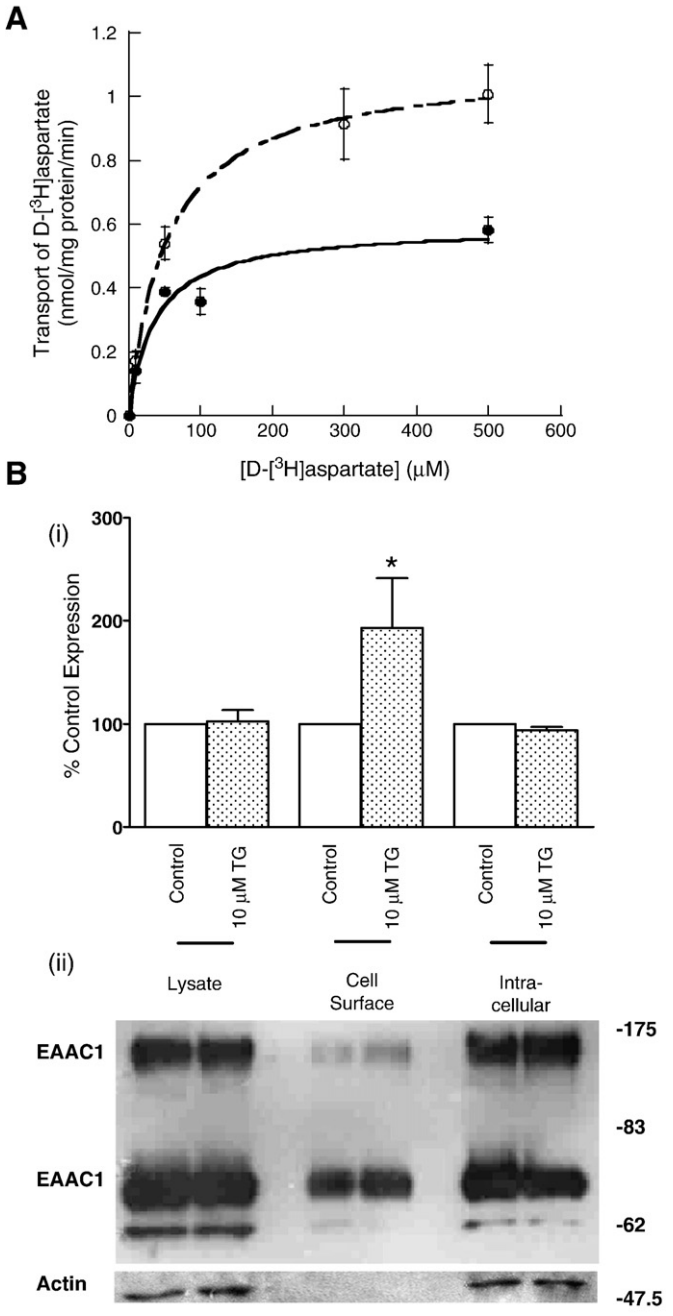

Fig. 3. Thapsigargin-induced changes in functional activity and expression of EAAC1 in C6 glioma cells. (A) Sodium-dependent transport of D-[^{3}H]aspartate (10 – 500 μM) was determined in C6 glioma cells in both the presence (○) or absence (●) of 10 μM thapsigargin for a pre-incubation period of 15 min prior to initiation of the transport assay. The results are the mean ± SEM of at least 3 separate determinations measured in triplicate. (B) Cells were incubated in the absence (clear bars) or presence (shaded bars) of 10 μM thapsigargin for 15 min and expression of EAAC1 was determined using a membrane impermeant biotinylation assay. (i) Summary of four independent experiments (mean ± SEM), presented as the sum of both monomers (75 kD) and multimers (175 kD) and expressed as the percentage of the control in total lysate, cell surface and intracellular protein fractions. (ii) Representative Western blot of EAAC1 and actin expression in each fraction. *$P<0.05$, Students unpaired t-test.

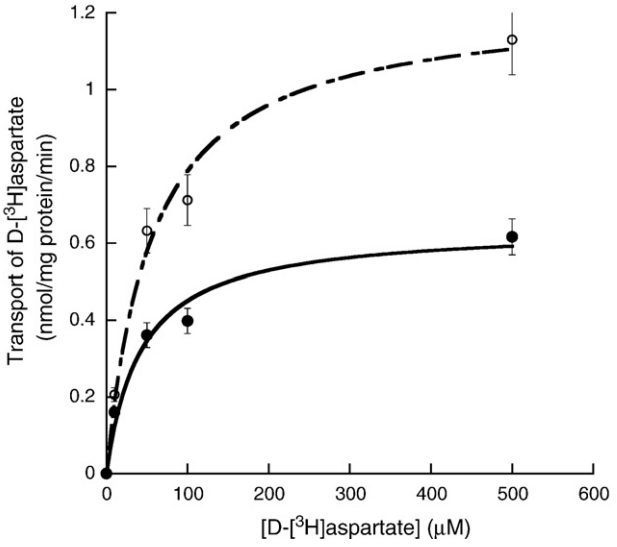

Fig. 4. Kinetic analysis of D-[^{3}H]aspartate transport in C6 glioma cells in the presence of phorbol myristate-3-acetate. Sodium-dependent transport of D-[^{3}H]aspartate (10–500 μM) was determined in C6 glioma cells in both the presence (○) or absence (●) of 100 nM PMA for a pre-incubation period of 15 min prior to initiation of the transport assay. The results are the mean±SEM of at least 3 separate determinations measured in triplicate.

abolished the increase in transport that had been observed in the presence of thapsigargin alone (180±5% of control with thapsigargin alone, compared to 106±12% of control with both thapsigargin and Bis-X; $P<0.05$). Pre-incubation of C6 glioma cells in with either the protein kinase A inhibitor, H-89 (10 μM) or the CaM kinase II inhibitor, KN-93 (8 μM) for 15 min in the presence of 10 μM thapsigargin had no effect on the stimulation of 50 μM D-[^{3}H]aspartate transport observed in the presence of thapsigargin alone (Fig. 6). None of the inhibitors tested had any effect on transport of D-[^{3}H]aspartate in the cells in the absence of thapsigargin. Bis-X had no effect on the decrease in transport by thapsigargin observed in the absence of calcium (results not shown).

3.6. Blockade of store-operated calcium channels inhibits transport of D-[^{3}H]aspartate in C6 glioma cells

2-aminoethoxydiphenyl borate (2-APB) is a non-selective inhibitor of store-operated calcium channels (SOCCS) [23] and SKF 96365 specifically blocks the NSCC-2 subtype of SOCC [24,25]. Either 2-APB (50 μM) or SKF 96365 (10 μM) were added to the incubation medium for 20 min and 1 h, respectively, before initiation of a transport assay. Both compounds significantly decreased uptake of D-[^{3}H]aspartate (50 μM) in the absence of thapsigargin (53±4% of control with 2-APB and 66±3% of control with SKF 96365; Fig. 7). No stimulation in D-[^{3}H] aspartate uptake by thapsigargin was observed when the cells were co-incubated with 2-APB and thapsigargin for 20 min prior to the transport assay (Fig. 7A). In contrast, the stimulation in D-[^{3}H] aspartate (50 μM) transport arising from pre-incubation of the cells with 10 μM thapsigargin persisted when the cells were incubated with 10 μM SKF 96365 for 60 min before the addition of thapsigargin (96±5% of control; Fig. 7B). It is concluded that the stimulation of transport by thapsigargin is dependent on entry of calcium via the NSCC-1 subtype of SOCC.

4. Discussion

This study provides important information on the responsiveness of C6 glioma cells to calcium-modifying agents and on the relationship between intracellular calcium and the functional activity and cell surface expression of the EAAC1 glutamate transporter in these cells.

As an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, thapsigargin is a widely used tool for depletion of ER calcium stores and consequent elevation of the intracellular calcium concentration. As predicted, pre-incubation of C6 glioma cells with thapsigargin produced a significant increase in intracellular calcium that, apart from the initial rise, was dependent on entry of calcium from the extracellular medium. C6 glioma cells do not contain voltage-sensitive calcium channels [26] and it follows that calcium entry must, in the absence of receptor stimulation, occur by store-operated calcium

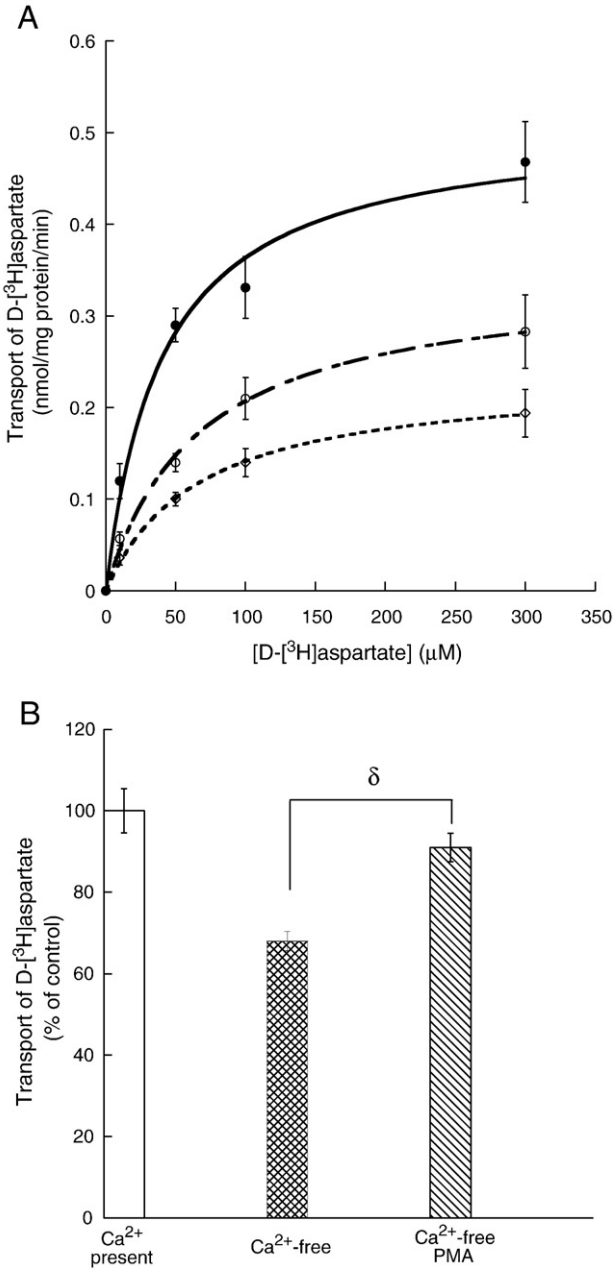

Fig. 5. Calcium-dependency of the stimulation of D-[^{3}H]aspartate transport in C6 glioma cells by thapsigargin and phorbol myristate-3 acetate. (A) Sodium-dependent transport of D-[^{3}H]aspartate (1–300 μM) was determined in both normal (●) and calcium-free Krebs' medium either with (◇) or without (○) prior incubation of the cells with 10 μM thapsigargin. (B) Transport of 50 μM D-[^{3}H]aspartate was determined in both normal and calcium-free Krebs' medium either with or without pre-incubation with 100 nM PMA. The results in (B) are expressed as the percentage of transport that occurred in normal Krebs' medium in the absence of PMA. All data are the mean±SEM of at least 3 separate determinations, each measured in triplicate. δ$P<0.05$.

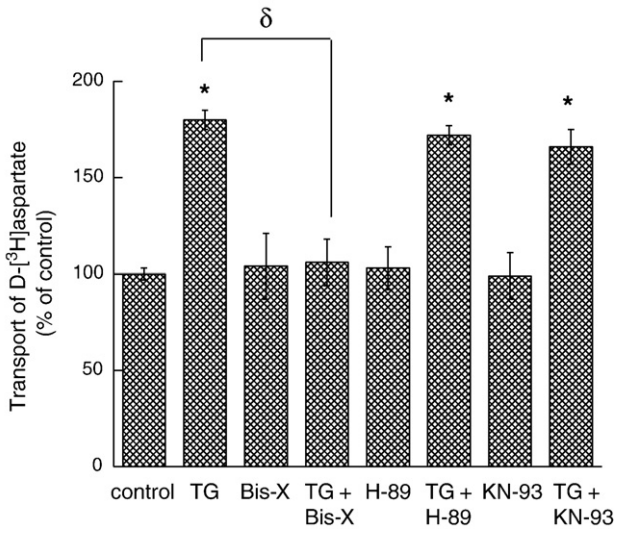

Fig. 6. The effect of protein kinase inhibitors on the stimulation of D-[^{3}H]aspartate transport by thapsigargin in C6 glioma cells. Cells were pre-incubated with 10 μM thapsigargin for 15 min prior to measurement of D-[^{3}H]aspartate transport in both the presence and absence of Bis-X (10 μM), H-89 (10 μM) or KN-93 (8 μM). The results are expressed as the percentage of transport observed in the absence of thapsigargin or inhibitors and are the mean ± SEM of at least 3 independent determinations measured in triplicate. *P<0.05, Student's t-test; δP<0.05, Neuman Keuls test.

channels (SOCCs). In C6 glioma cells, two types of SOCC operate (NSCC-1 and NSCC-2) [19]. SKF96395 inhibits NSCC-2 but not NSCC-1, whereas 2-APB is a non-selective inhibitor of both channels [23–25,27]. Therefore, since 2-APB inhibited the thapsigargin-mediated rise in intracellular calcium, whereas SKF 96365 did not, it is concluded that the increase in intracellular calcium occurs via the NSCC-1 subtype of SOCC in C6 glioma cells.

Pre-incubation of C6 glioma cells with thapsigargin significantly increased the rate of D-[^{3}H]aspartate transport, implying that a rise in intracellular calcium stimulates the EAAC1 transporter. The fact that the PKC inhibitor, Bis-X, blocked the stimulatory effect of thapsigargin and that incubation of the cells with PMA also increased transport, strongly suggests that the increase in transport observed with thapsigargin was mediated by PKC. These results are in agreement with other studies that have shown stimulation of EAAC1 by PKC in C6 glioma cells [14,15]. The increased cell surface expression of EAAC1 at the cell membrane following incubation of the cells with thapsigargin indicates that the observed increase in transport is due to increased delivery of the transporter to the plasma membrane. This result is in agreement with Bianchi et al. [14], who demonstrated an increase in cell surface expression of EAAC1 in C6 glioma cells by PKC. This group described the increased trafficking of EAAC1 to the membrane in response to PKC activation by phorbol esters as a 'peculiar feature of EAAC1' because PKC down-regulates the cell surface expression of the other major glutamate transporters, EAAT1 (GLAST) and EAAT2 (GLT1) [13].

A reduction in transport of D-aspartate was observed following incubation of C6 cells in either calcium free medium or in the presence of 2-APB. The 60% decrease in the V_{max} in EAAC1-mediated transport of glutamate in the absence of extracellular calcium is comparable to the reduced V_{max} recorded in two independent studies of the calcium-dependency of glutamate uptake in synaptosomes [28,29]. The conclusion in all cases is that extracellular calcium is required for optimal transport of glutamate. According to Mafra et al. [29], the reduced uptake of glutamate was not due to a de-stabilisation of membrane proteins caused by absence of calcium. One possibility may be that the decrease in uptake in calcium-free media may have been caused by partial activation of voltage-sensitive sodium channels, leading to a reduction in the sodium gradient required to drive the inward transfer of glutamate. We have not tested this directly, but note that tetrodotoxin reversed the reduction in transport in the absence of calcium in synaptosomes [28]. Another possible explanation, arising from our observation that 2-APB on its own reduced uptake of D-[^{3}H]aspartate is that entry of calcium from 'constitutively-active' calcium channels exerts a positive regulatory effect on EAAC1 in C6 cells, but the mechanism by which this might occur is not currently known. Our experiments suggest that it does not involve PKA, PKC, or CaMKII.

Removal of extracellular calcium or blockade of store-operated calcium entry by 2-APB in C6 glioma cells changed the response to thapsigargin from stimulation to net inhibition of D-[^{3}H]aspartate transport. The reduction in transport was kinase independent, since inhibitors of PKC, PKA or CaMKII had no effect on the decrease in transport by thapsigargin observed in calcium-free media. In the absence of extracellular calcium, therefore, thapsigargin produces the same effect in C6 glioma cells as it does in neuronal cultures expressing EAAC1 [11,30] and in primary astrocytes expressing GLAST [31]. The inhibitory pathway is kinase independent in

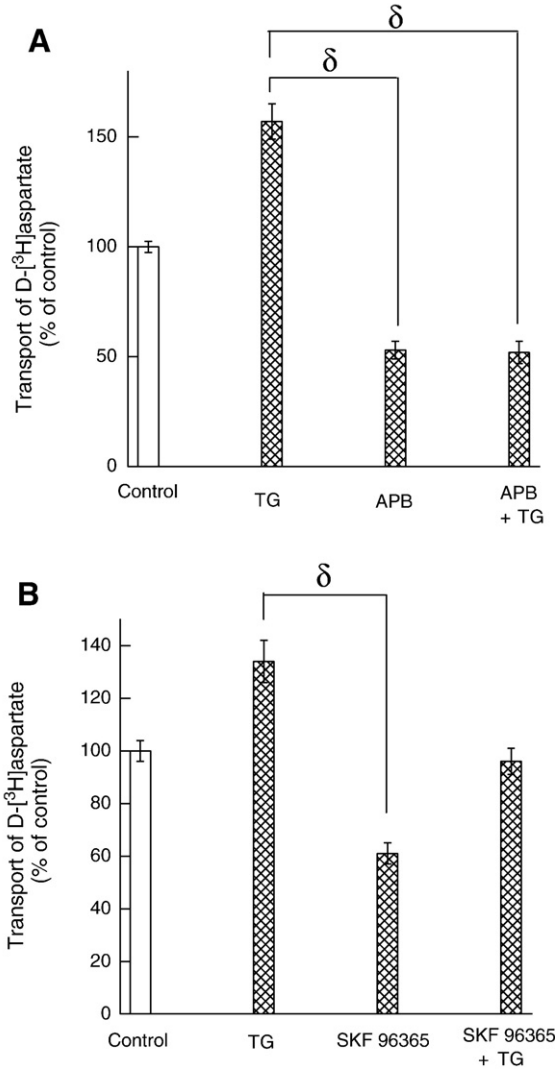

Fig. 7. The effect of SOC channel blockers on the stimulation of D-[^{3}H]aspartate by thapsigargin. C6 glioma cells were pre-incubated with either (A) 2-APB (50 μM, 20 min) or (B) SKF 96365 (100 μM, 1 h) before measurement of sodium-dependent transport of D-[^{3}H]aspartate. In each case, 10 μM thapsigargin was included for the final 15 min of the pre-incubation period. The results are expressed as the % of transport that occurred in the presence of thapsigargin alone and are the mean ± SEM of three separate determinations, each measured in triplicate. $^{\delta}P$<0.05, Neuman Keuls test.

neuronal cultures expressing EAAC1 and, unlike C6 cells, transport of glutamate is completely unaffected by PMA-induced stimulation of PKC [11].

We believe that C6 glioma cells are capable of two separate responses to the emptying of intracellular calcium stores by thapsigargin: one stimulatory, which requires entry of calcium from the extracellular medium, the other, inhibitory, which does not. We propose that in neuronal cells, such as were used in the study by Yang et al. [11], only the inhibitory pathway exists. Blockade of the thapsigargin-mediated stimulation of D-[^{3}H]aspartate transport and calcium influx by 2-APB, deemed a 'reliable blocker of store-operated calcium entry' [23], supports the theory of calcium entry from the extracellular medium as the crucial factor in determining whether thapsigargin stimulates, rather than inhibits, D-[^{3}H]aspartate transport in C6 glioma cells. The fact that the stimulation of D-[^{3}H] aspartate transport by thapsigargin persisted in the presence of SKF 96365 supports our view that calcium entry following depletion of ER stores is mediated by NSCC-1, but not NSCC-2. Absolute proof would require a selective inhibitor for NSCC-1, which is not currently available. The molecular link-up between store-operated calcium entry and the EAAC1 transporter requires further investigation. Both thapsigargin-mediated influx of calcium and PKC-dependent stimulation of EAAC1 do not require integrity of the actin cytoskeleton [14,32]. It is believed that a diffusible messenger forms a link between calcium stores and the plasma membrane calcium channels in the case of thapsigargin, whereas activation of purinergic receptors, for example, promotes a direct link-up between the calcium stores and the plasma membrane [32]. Other stimulators of EAAC1 activity in C6 glioma cells, such as endothelin-1 [20], increase cytoskeletal-dependent cell surface expression of the protein, which points to the existence of at least two pools of the EAAC1 transporter in C6 glioma cells — one that is linked to the cytoskeleton and another that is not.

In summary, these results have shown that store-operated calcium entry in C6 glioma cells stimulates EAAC1-mediated transport. Since C6 glioma cells are derived from a tumour cell line, the pathological relevance of this work needs to be considered. Astrocytoma and glioblastoma cell lines frequently differ from normal astrocytes in terms of their capacity for glutamate transport. The results presented in this report show that the responsiveness of glutamate transporters in C6 glioma cells to changes in intracellular calcium are also at variance with non tumour cells and suggest that it is not the calcium stores themselves, but rather store-operated calcium entry that ultimately controls transport. Further work needs to be directed towards understanding how the absence of a fully regulated glutamate transport system threatens glutamate homeostasis leading to glutamate-mediated cytotoxicity. Such knowledge would be instrumental to the development of strategies aimed at augmenting glutamate uptake in the diseased brain.

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