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RESEARCH PAPER

Etomidate reduces glutamate uptake in rat cultured glial cells: involvement of PKA

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Background and purpose: Glutamate is the main excitatory neurotransmitter in the vertebrate CNS. Removal of the transmitter from the synaptic cleft by glial and neuronal glutamate transporters (GLTs) has an important function in terminating glutamatergic neurotransmission and neurological disorders. Five distinct excitatory amino-acid transporters have been characterized, among which the glial transporters excitatory amino-acid transporter 1 (EAAT1) (glutamate aspartate transporter) and EAAT2 (GLT1) are most important for the removal of extracellular glutamate. The purpose of this study was to describe the effect of the commonly used anaesthetic etomidate on glutamate uptake in cultures of glial cells.
Experimental approach: The activity of the transporters was determined electrophysiologically using the whole cell configuration of the patch-clamp recording technique.
Key results: Glutamate uptake was suppressed by etomidate (3–100 μM) in a time- and concentration-dependent manner with a half-maximum effect occurring at 2.4 ± 0.6 μM. Maximum inhibition was approximately 50% with respect to the control. Etomidate led to a significant decrease of V_{max} whereas the K_m of the transporter was unaffected. In all cases, suppression of glutamate uptake was reversible within a few minutes upon washout. Furthermore, both GF 109203X, a nonselective inhibitor of PKs, and H89, a selective blocker of PKA, completely abolished the inhibitory effect of etomidate.
Conclusion and implications: Inhibition of glutamate uptake by etomidate at clinically relevant concentrations may affect glutamatergic neurotransmission by increasing the glutamate concentration in the synaptic cleft and may compromise patients suffering from acute or chronic neurological disorders such as CNS trauma or epilepsy.
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Abbreviations: DHK, dihydrokainate; EAAT, excitatory amino-acid transporter; GLAST, glutamate aspartate transporter; GLT1, glutamate transporter 1; TBOA, DL-threo-β-benzyloxyaspartic acid

Introduction

Glutamate is the principal excitatory transmitter in the vertebrate CNS. It is generally accepted that high extracellular glutamate concentrations lead to neuronal overstimulation and subsequent excitotoxic neuronal cell death (Meldrum, 2000). Increased extracellular levels of glutamate also seem to be involved in several human neurodegenerative disorders, such as Alzheimer's and Huntington's diseases, amyotrophic lateral sclerosis and epilepsy (Hediger 1999; Danbolt 2001).

As synaptically released glutamate is not inactivated by degradation, it must be removed from the extracellular space by effective transport systems. Five different excitatory amino-acid transporters (EAAT1–5) have been identified so far. Two of them, EAAT1 (glutamate aspartate transporter (GLAST)) and EAAT2 (glutamate transporter 1 (GLT1)) are predominantly expressed in astroglial cells, and the other three transporters in neurons (Beart and O'Shea, 2007). Although both glial and neuronal cells possess GLTs, the uptake of synaptically released glutamate occurs predominantly into glia (Rothstein *et al.*, 1996). The high capacity of glial cells for glutamate uptake is based on a rapid intracellular enzymic degradation of glutamate, which keeps the intracellular glutamate concentration at a low level.

During general anaesthesia the activity of the CNS is reduced by a modulation of the synaptic transmission such that either the excitatory systems are reduced or the inhibitory systems are enhanced (Little, 1996). At present, post-synaptic ion channels of neurons are believed to be main targets for general anaesthetics (Antkowiak, 2001), although there is increasing evidence that transporters of neurotransmitters located in extrasynaptic membranes of

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nerve and glial cells are involved as well. Various studies have shown that anaesthetics may affect the activity of GLTs in native cells in different ways (Miyazaki *et al*., 1997; Sugimura *et al*., 2001; Zuo, 2001). More recently, much effort has been devoted to the investigation of the effects of anaesthetics on the activity of specific types of GLTs after their heterologous expression in different cell systems (Do *et al*., 2002; Yun *et al*., 2006). A major result of these studies is that the effects of the anaesthetics may be mediated through intracellular signalling pathways. When reviewing these results the fact that intracellular signalling pathways affect specific types of GLTs in different ways depending on the cell type used for transfection should be borne in mind. Therefore, it is reasonable to study GLTs in their natural environment (Danbolt, 2001).

In this study, we focused on the anaesthetic, etomidate, which has previously been shown to reduce the activity of the neuronal EAAT3 by a PKC-dependent mechanism when expressed in oocytes (Yun *et al*., 2006). We show that etomidate also suppresses the activity of the glial GLTs that have a dominant function in glutamate uptake in the brain. Using cultures of undifferentiated glial cells that almost exclusively express GLAST we found that this is achieved by a PKA-dependent pathway.

Materials and methods

Preparation and culture of glial cells

The removal of rat brains for setting up glial cultures was approved by the local animal experimentation committee. Primary glial cultures were established from postnatal day 1 Sprague–Dawley rats (Charles River, Sulzfeld, Germany) as described elsewhere (Figiel and Engele, 2000). Briefly, cerebral hemispheres were removed under aseptic conditions and collected in ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline. Thereafter the tissue was incubated for 20 min in Dulbecco's phosphate-buffered saline containing 0.1% trypsin and 0.02% EDTA. Trypsination was terminated by transferring the tissue to Hank's balanced salt solution (HBSS, Gibco, Karlsruhe, Germany) supplemented with 10% foetal calf serum in which tissue pieces were gently dissociated by trituration through a plastic pipette. The resulting cell suspension was centrifuged for 5 min at $400 \times g$, and the pellet was resuspended in Dulbecco's modification of Eagle's medium supplemented with 10% horse serum (Gibco). Cells were plated on poly-D-ornithine (0.1 mg mL^{-1}; MW 30–70 kDa; Sigma, Deisenhofen, Germany). On reaching confluency, the cultured cells were trypsinized and replated. All experiments were performed with cells after the third passage. At this stage more than 90% of the cells are immunoreactive for glial fibrillary acidic protein (Figiel and Engele, 2000).

Electrophysiological experiments and data analysis

Electrophysiological measurements were performed 4–8 days after the final splitting. We applied the whole cell configuration of the patch-clamp technique (Hamil *et al*., 1981). The equipment consisted of an EPC-9 amplifier and TIDA software (HEKA, Lambrecht, Germany). Patch pipettes were drawn from borosilicate glass, with a pipette resistance of 4–5 MΩ when filled with (in mM): 130 KCl, 2 Na_2-ATP, 2 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 7.2.) To improve sealing, the electrodes were briefly dipped into dimethylsilan (2% in methylene chloride). During recording of membrane currents, glial cells were voltage clamped at −80 mV according to their resting membrane potential (McKhann *et al*., 1997). The cells were superfused with control solution (in mM) 140 NaCl, 2.7 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 6 glucose, and 12 mM HEPES (pH 7.3) at a rate of 4.5 mL min^{-1}.

Quantification of the electric charge (Q) was obtained by integrating the area under curve in the current (I) versus time (t) plot between the beginning and the end of substance addition ($Q = I \cdot t$).

To determine the IC_{50} of etomidate, the data were fitted (SigmaPlot 10, Systat Software, Chicago, IL, USA) using the Marquardt–Levenberg algorithm to an empirical Hill equation as described previously (Chen and Lipton, 2005):

$$I = 100 - \frac{I\text{max}}{(1 + (\text{IC}_{50}/(\text{etomidate}))^{\text{N}}}$$

where I is $I_{\text{etomidate}}/I_{\text{control}}$, I_{max} is the corresponding maximum, IC_{50} is the apparent 50% inhibition constant, (etomidate) represents the concentration of etomidate and N is the empirical Hill coefficient.

Immunoblotting

For immunoblot analysis the cultured glia were lysed by ultrasound in 60 mM Tris–HCl containing 2% SDS and 10% sucrose. The lysate was centrifuged at $1000 \times g$ for 10 min. The supernatant was collected and membranes were pelleted at $100\,000 \times g$ for 1 h at 4 °C. The pellet was resuspended in serum-free N2 medium (Engele, 1998) to obtain a final protein concentration of 1 mg mL^{-1}. Protein contents of both cell lysates and membrane fractions were determined with the BCA protein estimation kit (Pierce Chemical, Rockford, IL, USA).

Cell lysates were diluted 1:1 in sample buffer (250 mM Tris–HCl, pH 6.8, containing 4% SDS, 10% glycerol and 2% β-mercaptoethanol) and denatured at 95 °C for 5 min. Proteins (5 mg per lane) were separated by SDS–10% polyacrylamide gel electrophoresis and transferred to nitrocellulose by electroblotting. Nonspecific binding sites were blocked with 5% nonfat milk for 30 min, and then the blots were incubated overnight at 4 °C with one of the following antibodies: anti-GLT1 (1:4000; Chemicon, Temecula, CA, USA) or anti-GLAST (1:1000; Chemicon). The immunoreaction was detected with the enhanced chemiluminescence kit (Amersham Biosciences, Piscataway, NJ, USA). In all experiments, protein loading was controlled by staining the blots with actin antibodies (1:1000; Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Drug application

To ensure a localized drug application, a combination of two perfusion systems was installed: (i) a global bath perfusion with the inflow kept constant at 4.5 mL min^{-1}. and an

outflow that removed any excess fluid, and (ii) a local bath perfusion that generated a continuous fluid stream containing either just the control solution or the test substances at the desired concentrations. The local inlet was positioned at a distance of 50–100 μM upstream and the local outlet at about 300 μM downstream of the measuring field. All agents were applied to the cells through the local inlet at a rate of 0.8–1 mL min^{-1}. Selection of the different test solutions (kept under constant air pressure (MPCU-3; Lorenz, Lindau, Germany)) or syringes (driven by pumps (perfusor compact; B/Braun, Melsungen, Germany)) was controlled by magnetic valves.

Drugs were tested either by co-application with glutamate (DL-threo-β-benzyloxyaspartic acid, (TBOA), dihydrokainate (DHK), and etomidate (100 μM each)), after short-term preincubation (GF 109203X (20 μM), H89 (10 μM), chelerythrine (10, 50 μM), etomidate (3.1, 6.2, 12.5, 100 μM), staurosporine (0.2, 1.0 μM) as indicated in the figures) or after preincubation for 1 h (cytochalasin B (100 μM)). Drug concentrations were chosen according to recent reports about GLT systems (Duan *et al*., 1999; Gonzalez *et al*., 2002; Guillet *et al*., 2005; Fang *et al*., 2006; Yun *et al*., 2006).

Nomenclature

Drug and molecular target nomenclature conforms to the Guide to Receptors and Channels of the *British Journal of Pharmacology* (Alexander *et al*., 2008).

Statistical analysis

To present comparable results we only combined data which have been acquired from the same cell preparation and recorded on the same day. Results were reported as mean value ± s.d. and differences among groups were analysed by Student's *t*-test or ANOVA, followed by a Bonferroni post test. A difference between means was considered significant when $P<0.05$.

Chemicals

Unless otherwise stated all cell culture reagents were from Life Technologies (Karlsruhe, Germany). Etomidat-Lipuro was from B/Braun (Melsungen, Germany), Hypnomidate was from Cilag-Janssen (Neuss, Germany). All other drugs were purchased from Sigma (Deisenhofen, Germany).

Results

Characterization of GLT currents

Cultivated glial cells responded to rapid application of glutamate with a fast rising inward current that tended to plateau after 1–2 s. Upon removal of the transmitter, the current always rapidly decayed to zero. The amplitude of the response depended on the glutamate concentration, half-maximum activation occurring at $K_m = 8.8 \pm 2.3$ μM glutamate ($n = 7$ cells; Figure 1a). Repeated application of the same glutamate concentration resulted in almost identical responses.

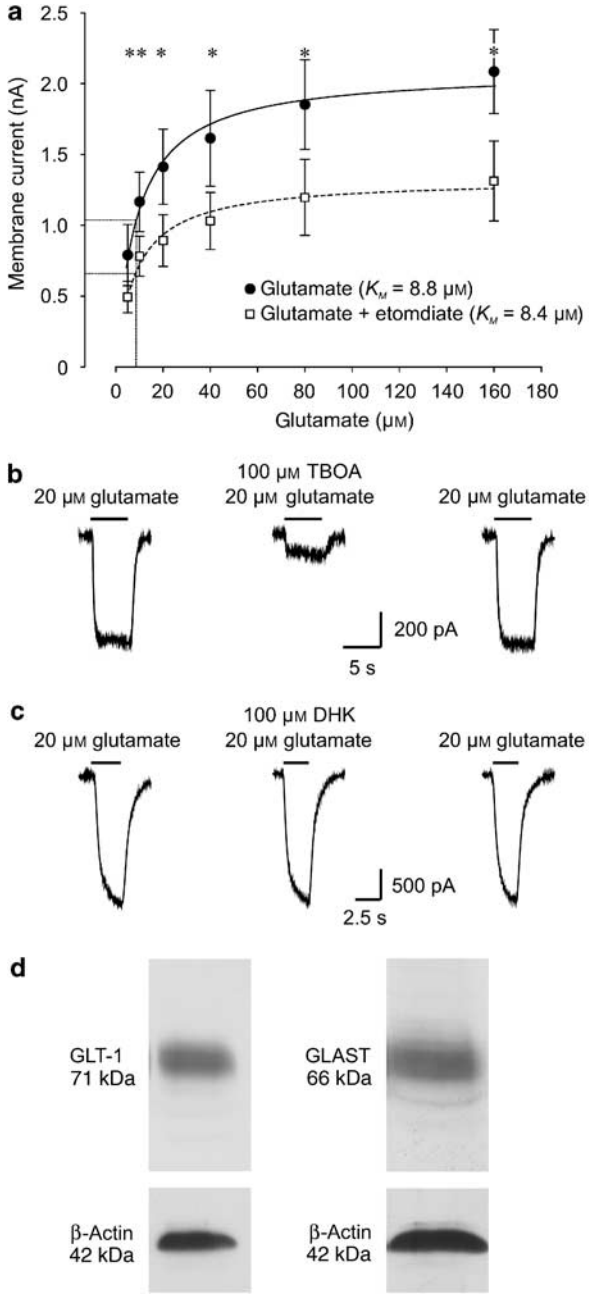

Figure 1 Characterization of the glial glutamate transporters (GLTs). (**a**) Dose–response curve of glial GLTs for increasing concentrations of glutamate + control solution (solid line) and glutamate + 12.5 μM etomidate (dashed line); 5–160 μM glutamate; $n = 6$ cells; Results are presented as means ± s.d.mean $^*P<0.05$, comparing control solution versus 12.5 μM etomidate on each glutamate concentration (5–160 μM) tested. (**b**) A typical current trace induced by application of 20 μM glutamate is shown on the left hand side. Immediate (center) and reversible (right hand side) suppression of glutamate transport by 100 μM DL-threo-β-benzyloxyaspartic acid (TBOA). Time interval between individual applications was 10 s. Bars denote the time of drug application. (**c**) Same protocol as in (**b**) using 100 μM dihydrokainate (DHK), a selective blocker of GLT1. (**d**) Third passage cortical glia cells were immunoblotted with anti-GLT1 antibodies (1:4000) or anti-GLAST antibodies (1:1000). In each lane 5 mg of total protein was loaded. Loading was controlled by staining the blots with actin antiserum.

To test for the specificity of the response, we added TBOA, a known blocker of the glutamate transport system (Vandenberg *et al*., 1997; Shimamoto *et al*., 1998), at a concentration of 100 μM to a 20 μM glutamate test solution (Figure 1b). As expected, the response was immediately reduced to 17.6 ± 6.8% with respect to the control ($n = 12$ cells; $P < 0.05$). The effect of TBOA was promptly reversible upon washout of the blocker. Application of 100 μM DHK, a selective blocker of GLT1 (Arriza *et al*., 1994), did not significantly ($P = 0.92$) reduce glutamate-evoked currents compared with the control (remaining current: 99.64.3%; $n = 19$ cells; Figure 1c).

To describe the transporter protein types involved in the cultures of rat glia, we studied expression levels of both glial transporters by immunoblot analysis, using GLT1- and GLAST-specific antibodies (Figure 1d). In freshly prepared cell lysates the GLT1 (1:4000) and GLAST (1:1000) antiserum each recognized a single protein with apparent MW of 71 and 66 kDa, respectively. Protein loading of the lanes was controlled by staining the blots with actin antiserum. The results demonstrate that the glial cells we used contained both glial transporter proteins, GLAST and GLT1.

Inhibition of glutamate uptake

In contrast to TBOA, 100 μM etomidate co-applied with 40 μM glutamate did not seem to influence the glutamate uptake immediately. The fast rise of the glutamate responses in the absence and presence of etomidate reached the same level. However, the longer etomidate was applied, the more the response decayed, an effect that was never observed in the absence of etomidate. Responses with ongoing decay could be observed over at least 25 s (Figure 2a).

To study whether etomidate influences the GLT even in the absence of glutamate, we applied 3–100 μM etomidate for variable time spans before eliciting the glutamate test response (Figures 2b–e). With preincubation, etomidate always reduced the immediate magnitude of the glutamate response. The ongoing decrease of the response observed with co-application of etomidate was never observed with preincubation of the drug (Figure 2b versus a).

The reduction of the response was clearly concentration-dependent. As a steady state was reached after approximately 3 min (Figure 2c), a half-maximum effect of the blocker (IC_{50}; cf. Chen and Lipton, 2005) could be determined to be at 2.4 ± 0.6 μM etomidate (40 μM glutamate; $n = 15$–21; Figure 2d). Furthermore, the reduction of glutamate uptake was completely reversible within about 60 s (Figures 2e and 4b), even for the highest concentrations tested (100 μM etomidate).

The decrease in current amplitude was not achieved by a change in glutamate affinity, as the K_m values in the absence (8.8 ± 2.3 μM) or presence of etomidate (8.4 ± 2.1 μM) were unaltered ($n = 7$ cells, Figure 1a).

These results are independent of the solvent of the etomidate preparation. No significant difference could be observed between etomidate dissolved in a lipid emulsion (Etomidat-Lipuro, B/Braun) and etomidate dissolved in propylene glycol (Hypnomidate, Janssen-Cilag) ($n = 7$ cells, 40 s preincubation; 40 μM glutamate; not shown). Furthermore, propylene glycol itself failed to affect glutamate uptake ($n = 4$ cells; 40 s preincubation; 40 μM glutamate; not shown).

Regulation and mechanisms of etomidate-mediated effects

As the inhibitory effect of etomidate on glutamate transport activity developed in a time-dependent manner, we assumed that etomidate exerted its effect in an indirect way. To test the possible involvement of PKs, we first analysed activators and blockers of PKC, which have previously been shown to affect transport activity of the related neuronal GLT EAAT3 (Yun *et al*., 2006).

Application of 2.5 μM phorbol-12-myrisate-13-acetate, an established PKC activator (Meijer, 1995), neither significantly affected the glutamate uptake by itself nor the inhibitory effect of etomidate on the transporter activity ($n = 4$ cells in each group, not shown).

Staurosporine, a well-known cell permeable broad spectrum inhibitor of PKs (Buchholz *et al*., 1991; Yanagihara *et al*., 1991; Meijer 1995; Murphy *et al*., 1999) did not evoke any currents by itself but decreased the glutamate uptake into glial cells at concentrations of 0.2 μM (78.6 ± 6.1%; $n = 5$ cells, Figure 3a) and 1.0 μM (80.6 ± 5.4%; $n = 5$ cells). Although the application of 12.5 μM etomidate by itself led to a reduction up to 80.0 ± 9.6% ($n = 8$ cells, Figure 3a), co-application of staurosporine with 12.5 μM etomidate diminished glutamate uptake to 60.9 ± 4.4% (0.2 μM staurosporine; $n = 5$ cells, Figure 3a) and 62.2 ± 15.0% (1.0 μM staurosporine; $n = 6$ cells). It should be note that there is no statistical difference of treatment effects comparing 0.2 and 1.0 μM staurosporine.

Blockade of PKC by means of chelerythrine decreased the glutamate uptake at concentrations of 10 μM (85.1 ± 7.7%; $n = 9$ cells) and 50 μM (69.8 ± 6.9%; $n = 12$ cells, Figure 3a). Co-application of the PK blocker with etomidate led to a decrease of the glutamate uptake up to 70.3 ± 10.2% (10 μM chelerytrine; $n = 9$ cells) and—significantly different from this result—54.9 ± 6.9% (50 μM chelerytrine; $n = 8$ cells, Figure 3a).

Finally, we tested GF 109203X another well-known broad spectrum inhibitor of PKs (Buchholz *et al*., 1991; Yanagihara *et al*., 1991; Meijer 1995; Murphy *et al*., 1999), and H89, a selective blocker of PKA (Lochner and Moolman, 2006). Both drugs did not evoke any currents by themselves but similarly enhanced the glutamate uptake in a time- and concentration-dependent manner. The inhibitory effect of 12.5 μM etomidate was completely suppressed when co-applied with either 10 μM H89 or 20 μM GF 109203X (Figure 3b, $n = 6$ cells).

As etomidate led to a fast decrease in glutamate uptake based on a decrease in transport capacity (v_{max}) we tested whether this may be achieved by a translocation of transporter proteins from the cell surface. For this purpose we used cytochalasin B, an inhibitor of actin polymerization, which has previously been shown to block an induced increase in cell surface expression of GLAST (Adolph *et al*., 2007).

To verify the sufficient inhibition of actin polymerization by cytochalasin B under the described conditions we showed that 60 min of preincubation of 100 μM cytochalasin B

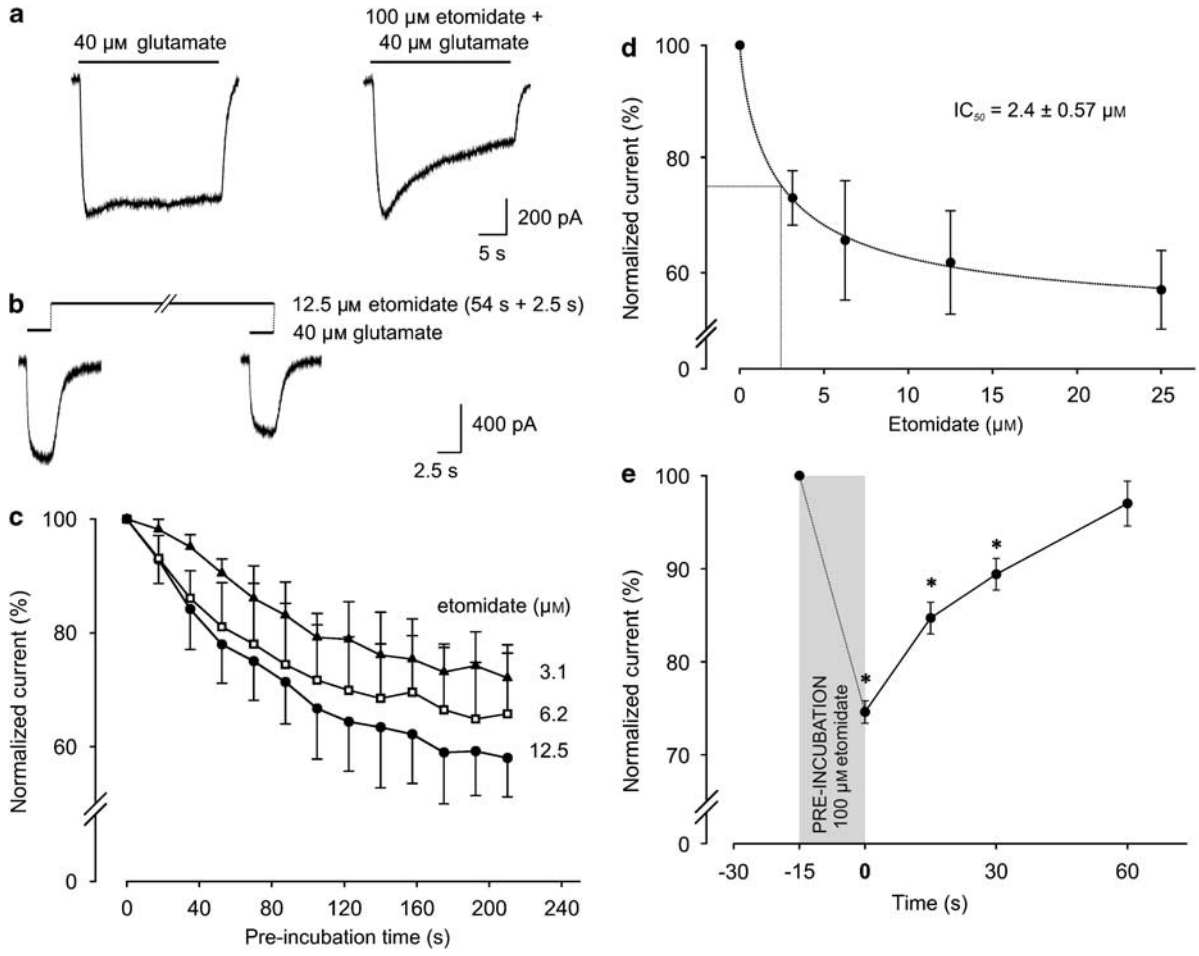

Figure 2 Reduction of glutamate uptake by etomidate. (**a**) A total of 40 µM glutamate was superfused for control and in combination with 100 µM etomidate. Etomidate time-dependently suppresses the glutamate transport activity. (**b**) A total of 40 µM glutamate was superfused for control and after preincubation with 12.5 µM etomidate for 54 s. (**c**) Summary of the effects of etomidate in clinically relevant concentrations (3.1–12.5 µM) on glutamate uptake (40 µM glutamate). Glutamate uptake decreases with the concentration of etomidate and the preincubation time. Data were obtained as shown in Figure 2b. Results are presented as means ± s.d.mean ($n = 5$–9 cells). Even the results for the lowest concentration tested (3.1 µM) become statistically significant (compared with control) when the substance is applied for >36 s. When comparing treatment effects accumulated over the whole observation period (0–225 s) using ANOVA for repeated measures, control versus 3.12, 3.12 versus 6.25, and 6.25 versus 12.5 µM are significantly different ($P<0.05$; $n = 65$–91). (**d**) As the effect of 3.1, 6.2, 12.5 and 25 µM etomidate reached a steady state after approximately 3 min, an IC_{50} of 2.4 ± 0.6 µM etomidate could be calculated (190–225 s preincubation, 40 µM glutamate). Comparing treatment effects, control versus 3.12, 3.12 versus 6.25, and 12.5 versus 25 µM are significantly different ($P<0.05$; $n = 15$–21). (**e**) The inhibitory effect of 100 µM etomidate (preincubation time 15 s) is completely reversible upon washout (40 µM glutamate). Results are presented as means ± s.d.mean ($n = 6$ cells). *$P<0.05$, compared with control.

almost completely abolished the increase in glutamate uptake induced by the PKA-blocker H89 (10 µM for 90 s; 20 µM glutamate, Figure 4a). In this case the basal glutamate transport activity was not affected. After 30 min of recovery reapplication of 10 µM H89 for 90 s led to an increase of glutamate uptake up to 120% as could be observed previous to the application of cytochalasin B. This finding suggests a fast restoration of polymerization capacity.

In contrast, preincubation of glial cell cultures for 60 min with 100 µM cytochalasin B did not significantly affect the etomidate-induced decrease in glutamate uptake (77.8 ± 3.5% after 54 s of preincubation with 12.5 µM etomidate; $n = 5$ cells, $P = 0.92$; 40 µM glutamate, not shown). Furthermore, the recovery from etomidate treatment was not altered ($n = 5$ cells, 12.5 µM etomidate, 40 µM glutamate, Figure 4b).

Discussion

Characterization of GLT currents

We have shown that etomidate blocks the uptake of glutamate into glial cells cultivated from the rat cortex. It is suggested that the blocking effect on GLAST, the main active GLT of the cells investigated here, is mediated through aPKA-dependent pathway.

As the transport of glutamate is electrogenic (Danbolt, 2001), we used the patch-clamp technique to analyse the effects of etomidate and various agents affecting intracellular signalling pathways on the activity of the GLT systems of cultured glial cells. As none of the tested substances (chelerythrine, cytochalasin B, DHK, etomidate, GF 109203X, H89, staurosporine, TBOA) led to measurable currents in the absence of the transporter substrate

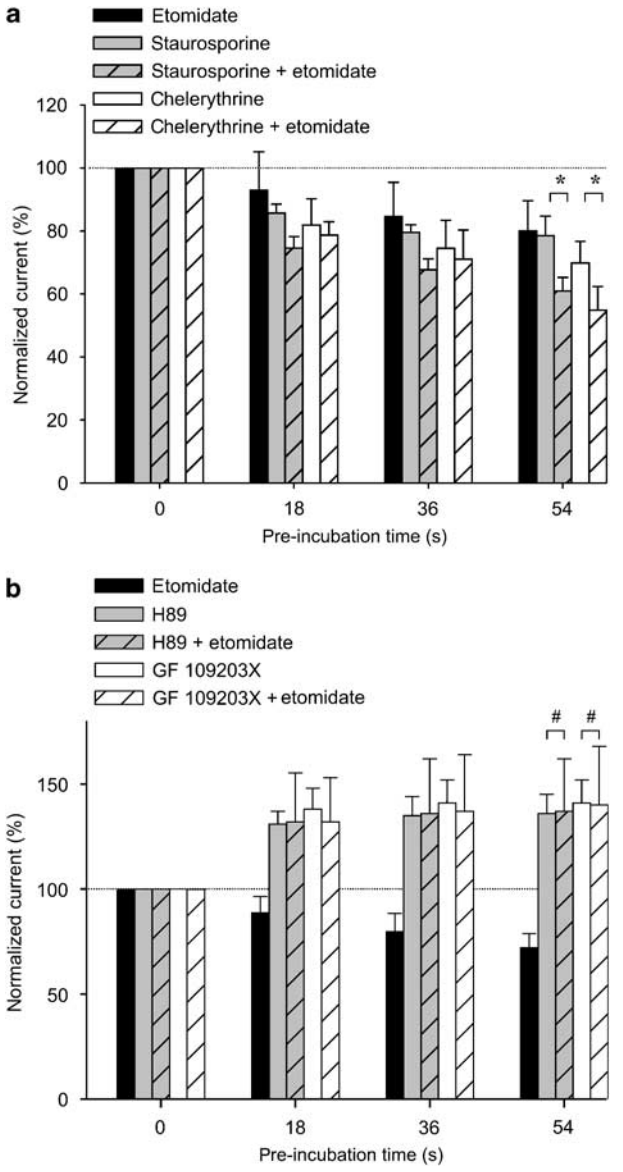

Figure 3 Effect of etomidate on glutamate uptake in the absence and presence of blockers of PKs. (**a**) PKC inhibition: the inhibitory effects of etomidate (12.5 μM), staurosporine (0.2 μM), and chelerythrine (50 μM) alone and in combination are illustrated. An additive effect without interference of etomidate and the PKC inhibitors can be observed. Results are presented as means ± s.d.mean ($n = 5$–8 cells; 40 μM glutamate). *$P < 0.05$, comparing staurosporine versus staurosporine + etomidate and chelerythrine versus chelerythrine + etomidate. (**b**) PKA inhibition: the inhibitory effect of etomidate (12.5 μM) and the enhancing effects of GF 109203X (20 μM), and H89 (10 μM) alone and in combination are illustrated. The stimulatory effect of the PK blockers GF 109203X and H89 abolishes the inhibitory effect etomidate completely. Results are presented as means ± s.d.mean ($n = 6$ cells; 40 μM glutamate). $P > 0.05$, comparing H89 versus H89 + etomidate and GF 109203X versus GF 109203X + etomidate.

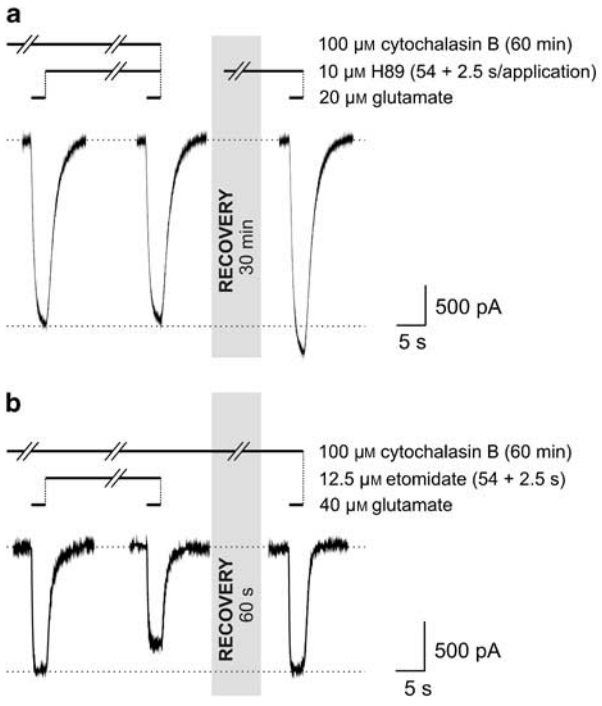

Figure 4 Actin network-related trafficking processes. (**a**) Blockage of H89-induced increase of glutamate uptake by cytochalasin B. Suppression of actin polymerization using cytochalasin B (100 μM for 60 min) almost completely abolished the increase in glutamate uptake induced by the PKA-blocker H89 through trafficking (10 μM for 90 s; 20 μM glutamate), whereas basal glutamate transport activity was not affected (center). After 30 min of recovery, reapplication of 10 μM H89 for 90 s led to an increase of glutamate uptake up to 120% (right). (**b**) Etomidate-induced decrease in glutamate uptake does not depend on trafficking of glutamate aspartate transporter. Preincubation of a similar preparation of glial cell cultures as used for (**a**) with cytochalasin B (100 μM, 1 h of preincubation) influences neither the inhibitory effect of etomidate (center; 12.5 μM, 54 s preincubation; 40 μM glutamate) nor the recovery time needed for reconstitution of the primary uptake capacity after etomidate treatment (right; 60 s of recovery; cf. Figure 2e).

glutamate, we suggest that the changes of the measured currents described for the tested substances are transporter-specific effects.

Using the non-competitive GLT blocker TBOA the measured currents could clearly be assigned to the activity of GLT systems (Vandenberg *et al*., 1997; Shimamoto *et al*., 1998; Beart and O'Shea, 2007). Furthermore, the elicited currents may be referred predominantly to the activity of GLAST, as no effect of the GLT1-selective blocker, DHK, could be demonstrated (Arriza *et al*., 1994). This is in agreement with previous investigations showing that cultured glial cells do not express active GLT1 as long as they are cultured in the absence of appropriate growth factors or neurons (Gegelashvili *et al*., 1996, 1997; Zelenaia *et al*., 2000; Leonova *et al*., 2001; Robinson 2002; Susarla *et al*., 2004).

Note that this statement is restricted to the activity of GLTs. As shown in previous studies (Figiel and Engele, 2000), glial cell cultures contain GLAST and GLT1. We have confirmed this result by an immunoblot using anti-GLT1 or anti-GLAST antibodies and showed that glial cultures cultivated as described contain both types of transporters. This is in accordance with the report of Vermeiren *et al*. (2005) describing expressed but inactive GLT1 proteins in primary cultures of rat cortical astrocytes. The authors discuss readily available GLT1 in submembrane compartments

or dormant cell-surface GLT1 that can be activated by post-translational modifications.

Regulation and mechanisms of etomidate-mediated effects

As the blocking effect of etomidate on the GLT system, GLAST, was not immediately obvious with the simultaneous application of glutamate, an indirect mechanism is suggested. This is further supported by the finding that the inhibition developed with time and did not require the presence of the transporter substrate. Etomidate and several blockers of PKs vary the activity of GLAST with a similar time course.

GF 109203X or staurosporine, two nonselective blockers of different PKs (Buchholz *et al*., 1991; Yanagihara *et al*., 1991; Meijer 1995; Murphy *et al*., 1999) enhanced or suppressed the activity of GLAST, respectively. As there is no statistical difference of treatment effects comparing 0.2 and 1.0 μM staurosporine, the effect of staurosporine on GLAST in cultured glial cells appears to be saturated already at a concentration of 0.2 μM. H89, a selective blocker of PKA (Lochner and Moolman, 2006), increased glutamate uptake by GLAST.

As the effects of staurosporine (at both concentrations tested) and etomidate each by itself are obviously additive, it is suggested that staurosporine and etomidate decrease the activity of GLAST through different mechanisms. The same is true for the PKC blocker chelerythrine. In contrast to the investigations on EAAT3 expressed in *Xenopus* oocytes (Yun *et al*., 2006), phorbol-12-myrisate-13-acetate, a PKC activator (Meijer, 1995), failed to influence the effect of etomidate. Therefore PKC seems to have a minor function mediating the suppression of glutamate uptake by etomidate.

GF 109203X and H89 enhance glutamate uptake in cultured glial cells through blockade of the PKA pathway (Adolph *et al*., 2007) and completely suppress the inhibitory effect of etomidate. Therefore, it is most probable that they use a common mechanism. From the selectivity of H89, it can be concluded that the inhibition of glutamate uptake caused by etomidate is mediated by the PKA pathway. It should be pointed out that the PKA-mediated process is essential for the transduction of the etomidate effect but there is also evidence that this process is just one among others, as 'pure' PKA activators such as forskolin (Adolph *et al*., 2007) failed to mimic the etomidate effect.

So far, there are different findings about the involvement of PKs in the regulation of GLTs. This is mainly because the effect of a particular PK does not only depend on the type of the transporter but also on the cell system used. Several groups studied the effects of PKC on GLT1 activity and obtained contradictory results showing that activation of PKC increases, decreases or has no effect on GLT1 activity using either HELA-, MDCK-, MCB or L-M(TK-) cells (Casado *et al*., 1991, 1993; Carrick and Dunlop, 1999; Tan *et al*., 1999). Similarly, contradictory results were obtained when the effects of PKC activation on GLAST activity were analysed (Casado *et al*., 1991; Gonzalez and Ortega, 1997; Daniels and Vickroy, 1999). These controversial findings are discussed in terms of cell type-specific sets of PK isoform and/or cell type-specific intracellular trafficking, which has an prominent function in the regulation of GLT systems (Sims and Robinson, 1999; Robinson, 2002). Thus, the use of cultured glial cells, instead of heterologous expression systems, more closely mimics the intracellular milieu *in vivo* (Schlag *et al*., 1998; Zelenaia *et al*., 2000; Leonova *et al*., 2001; Robinson, 2002).

The kinetic study showed that etomidate decreased the V_{max} but not K_m of glutamate uptake by GLAST, suggesting that etomidate reduces the available number or the turnover rate of GLAST rather than changing the affinity of GLAST for glutamate (Danbolt, 2001, Yun *et al*., 2006).

Glutamate aspartate transporter trafficking between intracellular compartments and the plasma membrane has been suggested to be a major regulatory mechanism of GLAST activity (Adolph *et al*., 2007). Actin is a major functional and structural cytoskeletal protein that mediates such diverse processes as motility, intracellular trafficking and control of cell shape (Howe, 2004). As shown in an efficacy study under similar conditions, the actin filament disrupter, cytochalasin B, sufficiently suppressed network-dependent trafficking processes of GLAST between intracellular pools and the cell surface induced by the PKA inhibitor H89.

As the effect of etomidate was not influenced by preincubation with cytochalasin B, the decrease in glutamate uptake is likely not to be based on an altered surface expression of GLAST in terms of an depletion of transporter proteins through trafficking. Furthermore, as the recovery time after etomidate treatment was unaffected by cytochalasin B, we conclude that trafficking is not necessary for the reconstitution of the primary uptake capacity in the form of protein replacement.

Excluding affinity changes and trafficking processes, we conclude that etomidate exerts its reversible effect by changing the turnover rate, through a PKA pathway.

Clinical correlates and relevance

Although a low level of extracellular glutamate is in general desirable for anaesthetic purposes, inhibition of the glial glutamate uptake may not necessarily compromise the process of anaesthesia. A moderate increase of glutamate in the synaptic cleft can equally desensitize postsynaptic glutamate receptors and/or inhibit presynaptic glutamate release (Tong and Jahr, 1994). Such a balanced effect of the blockade of glutamate uptake was recently shown for various glutamate receptor subtypes. AMPA- and kainate-mediated synaptic transmissions were suppressed whereas NMDA-mediated signalling was enhanced (Kidd and Isaac, 2000). Thus, the final consequences of an etomidate-induced blockade of GLTs during its use for clinical anaesthesia cannot easily be predicted from fundamental research on cell culture systems.

The maximum serum concentration of etomidate seen in clinical use is about 10 μM (Cold *et al*., 1986). Although it is almost impossible to deduce exact tissue concentrations in the CNS of the lipophilic etomidate (octanol/water partition coefficient = 1000; Levron and Assoune, 1990), from measurements in aqueous blood samples, this value is on the same order of magnitude as the concentration tested here,

which suppresses the glial glutamate transport by about 40%. Consequently, inhibitory effects of etomidate on glial glutamate uptake are likely to occur during anaesthesia. A manifestation of such inhibitory effects *in vivo* may be the etomidate-induced seizures in patients suffering from temporal lobe epilepsy. These focal-type seizures, recorded as fast activity in the EEG, are accompanied by increased extracellular glutamate levels after the epileptic fit (Proper *et al*., 2002; Tian *et al*., 2005; Yun *et al*., 2006). On account of decrease of the seizure threshold, etomidate has been found to be useful for electroconvulsive therapy (Conca *et al*., 2003) and for intraoperative mapping of seizure foci (Gancher *et al*., 1984). Similarly, etomidate-dependent inhibition of glial glutamate transport could be the cause for involuntary muscle movements, termed myocloni, which occur in about one-third of all patients during the induction of anaesthesia with etomidate. Although myocloni resemble seizures, they are not associated with epileptiform discharges in the EEG, and rather appear to result from a disinhibition of subcortical structures that normally suppress extrapyramidal motor activity (Doenicke *et al*., 1999; Khalil *et al*., 1999).

The effect of etomidate on glial and neuronal GLTs and their potential clinical consequences call for a cautious use of this anaesthetic in patients in which GLT action is compromised to avoid aggravation of disease-related symptoms and neurotoxic effects. This may be the case with several acute and chronic neurological disorders. The list of conditions includes stroke, CNS trauma and epilepsy, as well as Alzheimer's disease (Danbolt, 2001).

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Conflict of interest

The authors state no conflict of interest.

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