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Neuronal and Glial Glutamate Transporters Possess an SH-based Redox Regulatory Mechanism

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Abstract

Glutamate uptake into nerve cells and astrocytes via high-affinity transporters controls the extracellular glutamate concentration in the brain, with major implications for physiological excitatory neurotransmission and the prevention of excitotoxicity. We report here that three recently cloned rat glutamate transporter subtypes, *viz.* EAAC1 (neuronal), GLT1 and GLAST (glial), possess a redox-sensing property, undergoing opposite functional changes in response to oxidation or reduction of reactive sulphydryls present in their structure. In particular, thiol oxidation with 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) and disulphide reduction with dithiothreitol (DTT) result, respectively, in reduced and increased uptake capacity by a preparation of partially purified brain transporters as well as by the three recombinant proteins reconstituted into liposomes. In this model system, EAAC1, GLT1 and GLAST react similarly to DTT/DTNB exposures despite their different contents of cysteines, suggesting that only the conserved residues might be involved in redox modulation. Redox sensitivity is a property of the glutamate transporters also when present in their native cell environment. Thus, by using cultured cortical astrocytes and the whole-cell patch-clamp technique we were able to observe dynamic increase and decrease of the glutamate uptake current in response to application of DTT and DTNB in sequence. Moreover, in the same paradigm, DDT-reversible current inhibition was observed with hydrogen peroxide instead of DTNB, indicating that the SH-based redox modulatory site is targeted by endogenous oxidants and might constitute an important physiological or pathophysiological regulatory mechanism of glutamate uptake *in vivo*.

Introduction

Glutamate uptake in neurons and astrocytes maintains the extracellular glutamate concentration at the threshold of excitatory amino acid (EAA) receptor activation (Lipton and Rosenberg, 1994). Thereby, a high signal-to-noise ratio for excitatory neurotransmission is obtained and harmful receptor overstimulation is prevented. The role of reuptake in shaping EAA synaptic currents is presently under debate (Isaacson and Nicoll, 1993; Sarantis *et al.*, 1993; Barbour *et al.*, 1994; Tong and Jahr, 1994; Takahashi *et al.*, 1995; Wadiche *et al.*, 1995a), while impaired transport would contribute to neuronal damage in acute as well as long-term neuropathologies (Szatkowski and Attwell, 1994; Rothstein *et al.*, 1995; Rothstein *et al.*, 1996a). At least four transporter subtypes have now been cloned: *viz.* GLAST (Storck *et al.*, 1992), GLT1 (Pines *et al.*, 1992), EAAC1 (Kanai and Hediger, 1992) and EAAT4 (Fairman *et al.*, 1995). These transporters exhibit ~50% sequence identity and have different patterns of distribution in the brain (Kanner, 1993; Rothstein *et al.*, 1994; Chaudry *et al.*, 1995; Lehre *et al.*, 1995). The uptake process is accompanied by the co-transport of two or three Na^+ ions, the counter-transport of one K^+ ion, and either the co-transport of one H^+ or the counter-transport of one OH^- ion, leading to electrical and pH changes (Attwell and Mobbs, 1994; Zerangue and Kavanaugh, 1996). Moreover, chloride fluxes associated with transport have been recently described (Wadiche *et al.*, 1995b). Transporters are also biochemically regulated, e.g. via protein kinase C-mediated phosphorylation (Casado *et al.*, 1993) and arachidonic acid (Trotti *et al.*, 1995a; Zerangue *et al.*, 1995). We have recently found that glutamate uptake in astrocytes is reduced by reactive oxygen species (Volterra *et al.*, 1994). Inhibition probably derives from direct oxidation of the glutamate transporters at a conserved site. Thus, recombinant EAAC1, GLT1 and GLAST are all similarly inhibited by peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2) (Trotti *et al.*, 1996). Sulphydryl groups on cysteine residues are highly sensitive to oxidation. In many proteins their redox interconversions account for functional changes and represent a regulatory mechanism (Aizenman *et al.*, 1989; Ruppersberg *et al.*,

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1991; Petronilli *et al.*, 1994; Pan *et al.*, 1995). We here investigate whether the glutamate transporter family also is supplied with an SH-based mechanism conferring redox sensitivity. The present results have been reported previously in abstract form (Trotti *et al.*, 1995b; Volterra *et al.*, 1996).

Materials and methods

Materials

3-[(3-cholamido-propyl)dimethylammonio]-1-propanesulphonate (CHAPS), mercuric chloride ($HgCl_2$), *p*-chloromercuribenzoic acid (pCMB), *p*-chloromercuryphenylsulphonic acid (pCMPS), *N*-ethylmaleimide (NEM), dithiothreitol (DTT), 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), hydrogen peroxide, wheat-germ agglutinin (free lectin), valinomycin and nigericine were purchased from Sigma. L-[^{3}H]glutamate (50 Ci/mmol) was from Amersham; L-glutamate, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 2-amino-5-phosphonovalerate (APV) and (*RS*)-α-methyl-4-carboxyphenylglycine (MCPG) were from Tocris Neuramin (Bristol, UK)

Purification and reconstitution of brain glutamate transporters into liposomes

These were done as previously described (Danbolt *et al.*, 1990). Briefly, crude rat brain plasma membranes were solubilized with CHAPS and centrifuged. The supernatant was passed through a wheat-germ agglutinin–agarose column and the glycoproteins were eluted with *N*-acetylglucosamine. This fraction (named 'partly purified transporters') was enriched ~10-fold in the glutamate transporters/total protein ratio and contained immunoreactivity to the three rat brain glutamate transporters cloned to date: GLT1, GLAST and EAAC1 (Lehre *et al.*, 1995; Bjørås *et al.*, 1996; and N. C. Danbolt, unpublished observation). For reconstitution, the fraction was mixed with a reconstitution mixture consisting of phospholipid, cholate and salt (PCSM), incubated on ice and gel-filtered on spin columns equilibrated with potassium phosphate buffer (KPi) as described (Trotti *et al.*, 1995). The liposomes form spontaneously during this gel filtration and the KPi buffer becomes their internal medium. The protein content of the liposome suspension was calibrated at 40–80 μg/ml, making it unlikely that more than one protein exists in the same liposome.

Transfection of glutamate transporter plasmid cDNAs in HeLa cells and reconstitution into liposomes

Rat GLT1 (Pines *et al.*, 1992), GLAST (Trotti *et al.*, 1996) and EAAC1 (Bjøras *et al.*, 1996) clones were obtained as described previously. HeLa cells infected with vTF-7 recombinant vaccinia-virus (Fuerst *et al.*, 1986; Blakely *et al.*, 1991) were transfected with pT7–GLT1, pT7–GLAST or pT7–EAAC1 plasmids by means of DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N* trimethylammonium methylsulphate) (Boehringer Mannheim, Germany), following the manufacturer's instructions. Control cells were treated with DOTAP without plasmid. Membranes obtained from transfected and control cells (Trotti *et al.*, 1996) were resuspended with buffer containing 0.5 NaCl, 10 mM sodium phosphate (NaPi, pH 7.4) and 1.5% cholate, and reconstituted with PCSM using spin columns as above (6 mg protein/ml liposome suspension). The resulting uptake capacity was ~150- (GLT1), ~25- (GLAST) and ~50-fold (EAAC1) higher with respect to mock-transfected cells similarly reconstituted.

[^{3}H]Glutamate uptake assay in liposomes

Uptake of [^{3}H]glutamate into liposomes was measured using an inwardly directed Na^+ gradient in the presence of a negative membrane potential, as described (Danbolt *et al.*, 1990; Trotti *et al.*, 1995a). Briefly, liposomes diluted in 130 mM NaCl, 20 mM NaPi (pH 7.4) and 1% glycerol (PBSG) ± SH reagents were incubated for 15 min DTT, DTNB), 5 min (NEM) or 70 s ($HgCl_2$, pCMB, pCMPS) at 25°C and gel-filtered to remove the compounds. Upon recovery from spin columns, liposomes were diluted again with PBSG, and the uptake assay was started by addition of 1.4 μCi L-[^{3}H]glutamate (50 nM) + 2.8 μM valinomycin, and stopped 70 s later. For studies on uptake kinetics the assay was run for only 2 s (Trotti *et al.*, 1995a). At the end of assay, liposomes were collected on filters and counted for radioactivity.

Cell cultures for electrophysiological recordings

Cortical cell cultures, obtained from newborn rats (postnatal day 1 or 2) by mechanical dissociation, were plated at low density (1–2 × 10^5/ml) in 20% fetal calf serum (FCS) + Eagle's Minimal Essential Medium (MEM, ICN Flow, Costa Mesa, CA) and allowed to stand for 24–30 h. After this period, they were washed vigorously to select cells already adherent and kept for 1 more day in 10% FCS + MEM. After another wash they were immediately used for patch-clamp experiments. Although several cell types were present in the dishes, we easily identified and selected a specific population formed by microaggregates of type-1-like astrocytes. The astrocytic nature of these cells was confirmed by positive staining for glial fibrillary acidic protein (GFAP) (for procedure see Codazzi *et al.*, 1995). The same cells were positive for the glial glutamate transporter GLAST but negative for GLT1 (N. C. Danbolt *et al.*, unpublished result). Astrocytic microaggregates appeared roughly rectangular, measuring ~100–150 μm on the major axis and 75–100 μm on the minor axis. They closely resembled the glial microislands described by Bekkers and Stevens (1991) and by Mennerick and Zorumski (1994) with respect to electrical properties. In particular, the entire microaggregates could be reasonably voltage-clamped because the cells were extensively coupled (as seen by dye diffusion from the patch pipette). Thus, stepping the membrane voltage in one glial cell produced a similar step in the membrane potential of another glial cell on the opposite side of the microaggregate. Moreover, aggregates possessed physiological resting potential (–75/–80 mV), low input resistance (100–140 MΩ) and glutamate uptake currents of the order of 50 pA. Solitary type-1-like astrocytes were also successfully patched, but often had very small uptake currents (<20 pA). At later times in culture aggregates became too big and flat to allow electrical recording.

Patch-clamp recordings

Patch-clamp recordings were made in the whole-cell configuration using a List EPC7 patch-clamp amplifier (List Electronic, Germany) as described previously (Volterra *et al.*, 1994). Borosilicate patch pipettes had a resistance of 5 MΩ when filled with intracellular solution containing (mM): KCl, 125; NaCl, 5; $MgCl_2$, 2; EGTA–KOH, 5; ATP–Mg, 5; adjusted to pH 7.2 with KOH. Microaggregates, voltage-clamped at –80 mV and maintained at 23°C, were continuously superfused with the following extracellular solution (mM): NaCl, 140; KCl, 3.4; $CaCl_2$, 1.8; $MgCl_2$, 1; glucose, 10; HEPES, 5; adjusted to pH 7.4. 50 micromolar CNQX + 50 μM APV + 500 μM MCPG was also present in all solutions to block EAA receptor currents. In some experiments 5 mM Ba^{2+} was added to block background K^+ conductances (Volterra *et al.*, 1994). Redox agents or glutamate were locally applied onto selected cells via a fast six-barrel microperfusion apparatus. The input resistance of the cells was monitored by giving a series of steps (100 ms, 1 Hz for 10 s) from –80 to –90 mV during recording of the holding current between glutamate challenges.

Results

Partly purified and reconstituted glutamate transporters are sensitive to SH reagents

The presence of functionally relevant sulphydryls in the glutamate transporters was investigated by testing the effects of several SH reagents on a preparation of partly purified rat brain transporters reconstituted into liposomes (see Materials and methods). [^{3}H]Glutamate uptake in the liposomes was variably inhibited by each of the tested agents, including membrane-permeant (pCMB, NEM) as well as membrane-impermeant (pCMPS) monothiol reagents and $HgCl_2$, which forms dithiol complexes. Dose–response curves were constructed for each compound (Fig. 1A). After identical exposure times (70 s), uptake was more potently inhibited by $HgCl_2$ [maximal inhibition (I_{max}), 100%; IC_{50}, 20 ± 3.5 μM, mean ± SEM, n = 6] than by pCMB (I_{max}, 75%; IC_{50}, 71±6 μM, n = 5) or pCMPS (I_{max}, >80%; IC_{50}, 289 ± 54 μM; n = 4). NEM also inhibited uptake, but with weaker potency (I_{max}, 45%; IC_{50}, 161 ± 8 μM; n = 4) and after longer exposures (5 min). The effect of $HgCl_2$ was studied in detail. The specificity of the reaction of $HgCl_2$ (10 or 100 μM) with transporter thiols was confirmed by complete reversal of uptake inhibition with DTT (Fig. 1B). Kinetic analysis of reconstituted transport in the absence of $HgCl_2$, gave a K_m of 9.6 ± 1.5 μM and a V_{max} of 10.2 ± 2 nmol/min/mg protein. Exposure to $HgCl_2$ (70 s, 10 μM) selectively reduced V_{max} (–36.6 ± 3%), leaving K_m unchanged (Fig. 1C).

SH groups confer redox-sensing property on glutamate transporters

We then asked whether reactive sulphydryls in the glutamate transporters undergo redox interconversions resulting in changes of uptake function. For this purpose we used two antagonistic redox reagents specific for SH groups, the thiol oxidant DTNB and the disulphide reductant DTT (van Iwaarden *et al.*, 1992). Freshly reconstituted transporters were exposed in parallel to control buffer, 3 mM DTT or 500 μM DTNB (15 min). [^{3}H]Glutamate uptake in the three groups was then compared; the result showed that the liposomes treated with dithiothreitol incorporated more glutamate than controls (mean ± SEM, +21.1 ± 2.6%; n = 21), while those exposed to DTNB incorporated less (–23 ± 2.1%; n = 21). If the same pool of liposomes was treated in two steps, first with DTNB and then with DTT, it took up less [^{3}H]glutamate than controls in the first step (–17.3 ± 2.3%, n = 3) but more than controls in the second (+12.7 ± 6.5%). In any single batch of liposomes that we tested, we observed uptake potentiation with DTT and inhibition with DTNB. However, the extents of the two opposing effects varied significantly among batches and always in a related manner, i.e. when one of the two was larger than average, the other was smaller (upper part of Table 1). An interdependent shift in DTT and DTNB potencies was also observed within the same liposomal preparation, at increasing times from reconstitution (0, 72 or 144 h). Thus, DTT became progressively more effective while DTNB became less effective (lower part of Table 1).

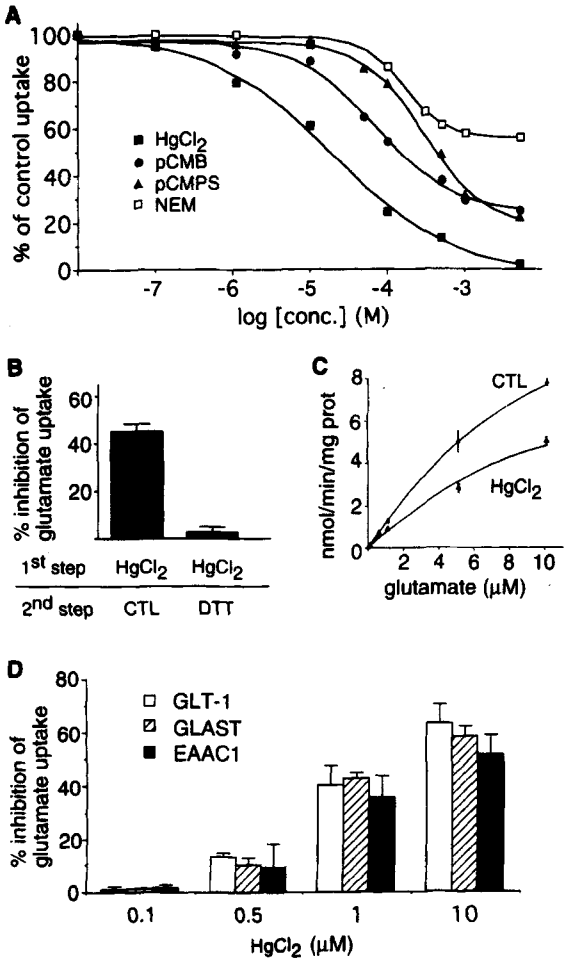

FIG. 1. Inhibitory effects of SH reagents on purified and recombinant glutamate transporters. Experiments in A, B and C are on partly purified transporters; the experiment in D is on recombinants. (A) Inhibitory potencies of SH reagents with distinct chemical properties: (■) $HgCl_2$; (●) pCMB; (▲) pCMPS; (□) NEM. See Materials and methods for protocols. Data points in the dose–response curves are mean values of at least four determinations in triplicate (SEM for any point is 10–20% of mean value). (B) DTT reverses $HgCl_2$ inhibition. First step: liposomes exposed (70 s) to 10 μM $HgCl_2$ and immediately gel-filtered to remove the agent. Second step: liposomes further incubated (15 min) before uptake assay, some with control buffer (CTL, to determine the extent of persistent inhibition), and others with 3 mM dithiothreitol (DTT). Data, expressed as percentage inhibition with respect to control (liposomes exposed in both steps to buffer alone), are the mean ± SEM of three experiments in triplicate. Complete reversal of inhibition was obtained also in one experiment with 100 μM $HgCl_2$. (C) Effect of $HgCl_2$ on uptake kinetics. Liposomes, in the absence (CTL) or presence ($HgCl_2$) of 10 μM $HgCl_2$, were incubated with different glutamate concentrations. Data are the mean ± SD of four experiments in quadruplicate. (D) $HgCl_2$ inhibits uptake by recombinant GLT1, GLAST and EAAC1. Recombinant transporters reconstituted in liposomes (see Materials and methods) were treated as in A. Bars represent the mean ± SEM of at least four experiments in triplicate. All inhibitions are significantly different from control except for the point at 0.1 μM (Student's t-test, two-tailed, $P < 0.01$).

Recombinant EAAC1, GLT1 and GLAST show identical redox sensitivity

Several transporter subtypes co-purify in the preparation used for the above studies, including EAAC1, GLT1 and GLAST (see Materials and methods). These proteins contain different numbers of cysteine residues (Kanner, 1993; Kanai *et al.*, 1994). Therefore, as the next step we tested the sensitivity of individual transporters to SH modifiers ($HgCl_2$) and SH redox reagents (DTT and DTNB). HeLa cells were transfected with cDNAs encoding the glutamate transporters EAAC1, GLT1 and GLAST and their cell membranes were reconstituted into liposomes to study effects on transport in isolation. As shown in Figure 1D, $HgCl_2$ inhibited uptake by each recombinant transporter dose-dependently and with similar potency. Moreover, all three of the transporters had their uptake capacity increased by DTT and

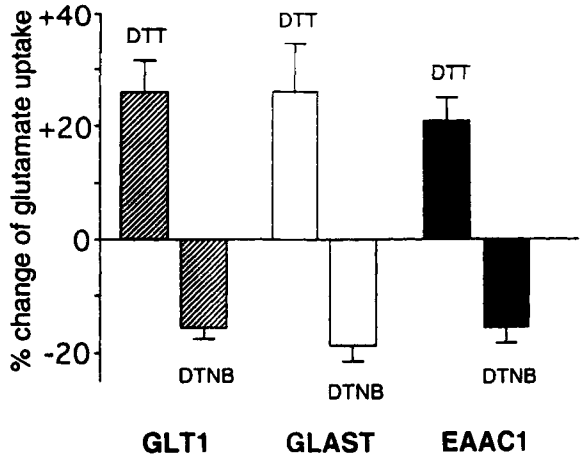

FIG. 2. Redox sensitivity of recombinant GLT1, GLAST and EAAC1. Liposomes containing the membranes of HeLa cells transfected with transporter cDNAs (see Materials and methods) were challenged with DTT and DTNB as in Table 1. Data represent percentage change with respect to control and are the mean ± SEM of four experiments in triplicate. Uptake by each of the three transporters was significantly modified by DTT (or DTNB); changes were not different among subtypes (one-way analysis of variance and Tukey method for multiple comparisons).

TABLE 1. Opposite effects of DTNB and DTT on partly purified glutamate transporters

	Change in glutamate uptake (%)	
	DTNB	DTT
Liposome preparation		
1	−28.9	+22.0
2	−44.0	+8.7
3	−23.4	+18.0
4	−34.9	+13.0
5	−7.0	+44.5
6	−40.1	+3.5
7	−16.1	+20.0
8	−6.4	+46.4
9	−29.5	+15.2
10	−0.5	+48.3
Time (h) after reconstitution		
0	−26.3 ± 3.8	+22.3 ± 1.9
72	−12.8 ± 2.9	+40.2 ± 1.7
144	−0.7 ± 1.5	+61.2 ± 5.0

Liposomes inlaid with freshly purified transporters were divided into aliquots, conserved at 4°C for later challenge, or immediately exposed (15 min) to 500 μM DTNB or 3 mM DTT, gel-filtered, and tested for [^{3}H]glutamate uptake (see Materials and methods). Data represent percentage change with respect to control. The upper part of the table reports the effects of DTT and DTNB in a representative number of separate preparations (each experiment is in triplicate, SEM omitted for clarity). The purpose is to emphasize the related variations in potency exhibited by DTT and DTNB in different batches of freshly reconstituted transporters. These variations probably reflect the different relative amounts of transporters present in the oxidized and in the reduced state in any given batch of liposomes. The lower part compares the effects of DTT and DTNB on single preparations at different times after reconstitution. Data are the mean ± SEM of six experiments in quadruplicate. Progressive increments in response for DTT and decrements for DTNB are all statistically significant (one-way analysis of variance and Tukey method for multiple comparisons).

decreased by DTNB, and the range of variation produced by the two agents was almost identical for each subtype (Fig. 2).

Dynamic redox modulation of astrocytic uptake current

To test whether glutamate transporters are redox-sensitive also when expressed in their native environment, we monitored the ionic current generated by the uptake process in cultured astrocytes and its responses to DTT/DTNB treatments. Microaggregates of type-1-like astrocytes were whole-cell voltage-clamped at –80 mV (for details see Materials and methods). Glutamatergic responses associated with uptake were selected by adding glutamate (30 μM) in the presence of a mixture of EAA receptor antagonists (50 μM APV + 50 μM CNQX + 500 μM MCPG). In ~60–70% of the cells, glutamate elicited a detectable inward current (mean ± SEM, –52 ± 4.9 pA; n = 25) that could be reversibly abolished by replacing Na^+ with choline in the bathing medium (see also Volterra *et al.*, 1994). Responses to glutamate application repeated at regular intervals (3 min) were consistent (mean variation of peak current, ±10–20%). However, if the cells were exposed to SH redox reagents during these intervals their subsequent responses to glutamate appeared to be profoundly changed, although these agents produced no important changes in the holding current and the input resistance of the cells. Exposure to dithiothreitol resulted in a significant increase in the uptake current (+80.6 ± 4.7%; n = 15). Conversely, DTNB decreased it to 61.4 ± 3.3% of control (n = 7). Dynamic increase and decrease of the current were produced by the two SH redox agents when added sequentially to the same cell (Fig. 3); any of them fully reversed the effect of the other, bringing the current to the expected potentiated or inhibited level. Results were identical in the presence or absence of 5 mM Ba^{2+}, which abolishes background K^+ conductances (see also Volterra *et al.*, 1994). Recordings obtained from a few isolated astrocytes showed very similar upward and downward modulation of the uptake current in response to dithiothreitol/DTNB, indicating that this was not due primarily to changes in the electrical coupling between the astrocytes. Interestingly, in some of the cells in which we failed to observe an initial response to glutamate, this appeared after exposure to DTT (–35.3 ± 6.7 pA; three of five tested cells).

Biological oxidants affect uptake current acting at redox SH groups

We then tested whether the effects of DTNB on electrogenic glutamate transport can be reproduced by oxidants formed during biological processes *in vivo*. For this study, we selected a mild oxidant that we have already found to directly interfere with glial uptake, H_2O_2 (Volterra *et al.*, 1994). Indeed, brief exposure (1 min) to 1 mM H_2O_2 produced significant reduction of the uptake current in naive cells (–47.4 ± 4%, n = 5). However, it also fully reversed current potentiation in cells previously exposed to DTT (Fig. 4). In either case, uptake current after H_2O_2 was reduced to the same level (on average, –33 pA), independently of its original level (–57 pA in naive cells versus –92 pA in DTT-treated cells). In the absence of other treatments this inhibited level was maintained in response to further glutamate challenges (not shown). However, re-exposure to DTT counteracted H_2O_2 inhibition, bringing the current back to ≈70% of the potentiated level and 31.8 ± 4.7% above the original control level (n = 7; Fig. 4).

Discussion

We here provide the first evidence that glutamate uptake is regulated by the chemical redox state of reactive sulphydryl groups present on the glutamate transporters. One neuronal (EAAC1) and two glial

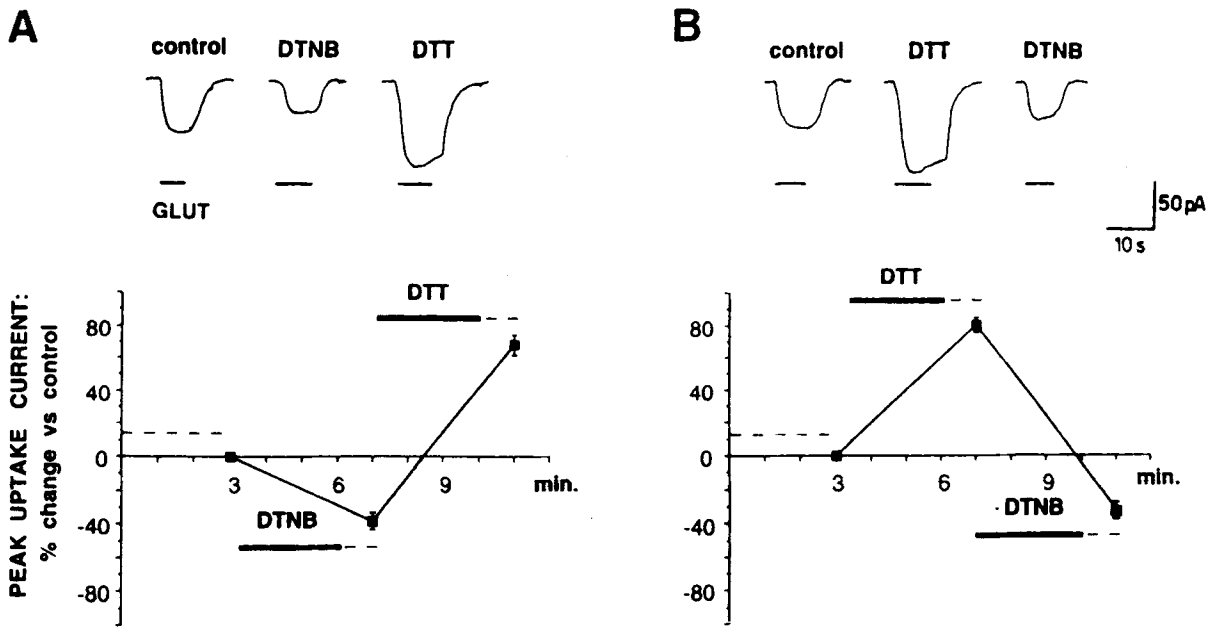

FIG. 3. Dynamic upward and downward modulation of glutamate uptake current in astrocytes by SH redox reagents. Astrocytic microaggregates were whole-cell clamped at –80 mV and the current responses to 30 μM glutamate (GLUT) in the presence of 50 μM APV + 50 μM CNQX + 500 μM MCPG were tested at regular intervals. In between, cells were exposed to SH-specific redox reagents (reduction, DTT, 3 mM; oxidation, DTNB, 500 μM) in the ox–red (A) or red–ox (B) sequence, without important changes in holding current and input resistance. Applications were followed by brief washout periods preceding glutamate challenges (dashed lines). The upper parts of panels A and B show inward currents elicited by glutamate in single representative experiments. The lower parts report the experimental paradigm and the mean percentage changes (±SEM) from the original peak uptake current produced by DTNB/DTT and DTT/DTNB treatments in seven recordings.

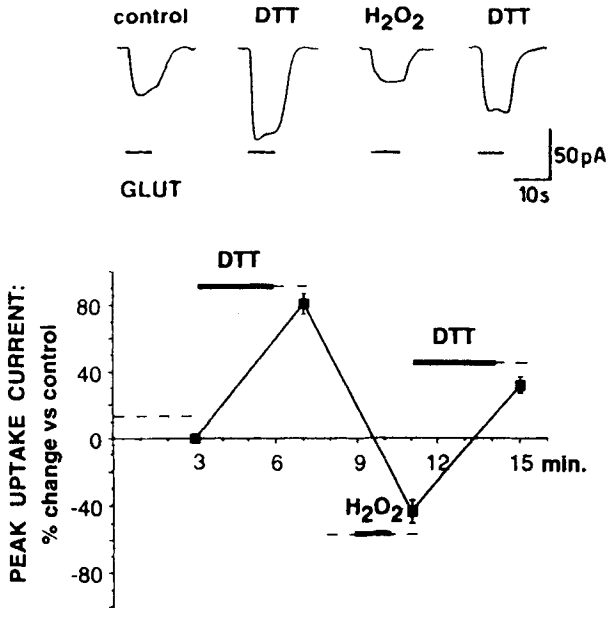

FIG. 4. Inhibition of glutamate uptake current by H_2O_2 involves an SH redox mechanism. Experimental conditions were as in Figure 3. H_2O_2 (1 mM) was applied for 1 min followed by 2 min of washout. DTT (3 mM) was applied for 3 min before and after H_2O_2. Upper part shows inward currents elicited by 30 μM glutamate in a representative experiment. Lower part shows the experimental paradigm and a diagram of average percentage changes from the original uptake current with sequential DTT–H_2O_2–DTT treatments in seven experiments. If control solution instead of H_2O_2 was applied between exposures to dithiothreitol, the potentiated current decreased only slightly (by <15%). H_2O_2 did not produce major changes in holding current and input resistance, as reported previously (Volterra *et al.*, 1994).

transporter subtypes (GLT1 and GLAST) all exhibit the redox-sensing property.

That sulphydryls could be crucial for glutamate uptake was suggested by the inhibitory effect of $HgCl_2$ in cultured astrocytes (Brookes, 1988) and in COS7 cells transfected with the glutamate transporters (Arriza *et al.*, 1994). Our data demonstrate that Hg^{2+} reacts directly with sulphydryl groups of the glutamate transporters, resulting in a reduced rate of uptake. Thus, $HgCl_2$ inhibition (i) is seen in a reconstituted system consisting of purified or recombinant transporter proteins incorporated into artificial membranes (Fig. 1A, D), and (ii) is fully reversed by DTT, a reagent specific for SH groups (Fig. 1B). Reconstituted transport is also inhibited by a number of monothiol reagents (pCMB, pCMPS, NEM), although less efficiently than by $HgCl_2$ (Fig. 1A).

Transporter thiols undergo redox changes, directly resulting in functional changes. Thus, after exposure to DTNB, which oxidizes free SH groups to disulphides, reconstituted transporters exhibit reduced uptake capacity. Conversely, they take up [^{3}H]glutamate at a higher rate than controls when pretreated with DTT, a disulphide-reducing agent (Table 1).

Recombinant EAAC1, GLT1 and GLAST reconstituted in liposomes do not show differences in their pattern of responses to $HgCl_2$ (Fig. 1D) or to the thiol redox reagents (Fig. 2), suggesting that they contain conserved redox-sensing elements. Structurally, the simplest model of a redox site predicts the presence of a pair of cysteines, existing as separate entities in the reduced state and held tightly together by a disulphide bond in the oxidized state (e.g. Sullivan *et al.*, 1994). Rat EAAC1, GLT1 and GLAST carry six, nine and three cysteine residues in their sequence respectively. Only two of them are conserved among the three transporters (Kanner, 1993; Biørås *et al.*, 1996) as well as in the human analogues EAAT1, EAAT2, EAAT3 and EAAT4 (Arriza *et al.*, 1994; Fairman *et al.*,

1995). One or both of these cysteines are strong candidates for being involved in the formation of a redox site. In the living brain, glutamate transporters might exist as homo-oligomeric complexes (Haugeto *et al.*, 1996). Therefore, redox sites could form either between cysteine residues within individual monomers or between cysteines bridging different monomers. Further studies, including site-directed mutagenesis, are required to distinguish among these possibilities and pinpoint the specific contribution of individual cysteines.

Upward and downward changes in response to DTT and DTNB respectively, are observed also in the glutamate uptake current recorded from cultured astrocytes (Fig. 3). Such changes most likely depend on a specific action of the two agents on the transport process. Thus, DTT and DTNB do not produce any relevant effect on the holding current or on the input resistance of the cells. Moreover, they modulate glutamate transport even in the presence of Ba^{2+}, which abolishes background K^+ conductances. These findings indicate that redox sensitivity is a property not only of the reconstituted transporters sitting in artificial membranes, but also of the native proteins, properly assembled and inserted in their cellular membranes (in particular, our preparation is highly immunoreactive for GLAST). Moreover, the electrophysiological experiments reveal that the uptake function adapts dynamically to the changes in the redox environment. Thus, when cells were exposed to DTNB and DTT in sequence, the uptake current shifted first below and then above control level (or *vice versa* if the treatments were reversed), indicating that transporter thiols interconvert reversibly between the oxidized and the reduced state. These redox interconversions represent a novel regulatory mechanism of the uptake function.

Natively, glutamate transport in cultured astrocytes shows a variable redox state, often between fully reduced and fully oxidized forms. Thus, in most cases, the uptake current responded to both DTT potentiation and DTNB inhibition. The full range of redox modulation observed at resting potential (V_h = –80 mV) was wide, going from –40% to +80% of control. Therefore, if operative *in vivo*, this mechanism might significantly influence the rate of glial uptake, with possible implications for phasic excitatory neurotransmission (Barbour *et al.*, 1994; Mennerick and Zorumski, 1994) as well as for the tonic control of the extracellular glutamate concentration. Remarkably, few cells showed an uptake current only after application of DTT, suggesting that oxidative inhibition could sometimes proceed until suppression of transport.

In the living brain, the redox state of glutamate uptake would depend on the balance between biological oxidants and reductants (e.g. the couple reduced/oxidized glutathione) or on the oxygen tension near the glutamate transporters. Shifts of these redox equilibria during normal brain activity might produce modulatory changes in glutamate transport, as observed for other signalling proteins (Ruppersberg *et al.*, 1991; López-Barneo, 1994).

On the other hand, the development of acute or chronic oxidant stress conditions, as reported in a number of brain pathologies, including transient ischaemia, trauma, amyotrophic lateral sclerosis and Alzheimer's disease, could bring the glutamate transporters to or maintain them in their most oxidized states, with reduced or even abolished uptake capacity. Indeed, impairment of glutamate uptake has been reported in the above pathologies (Silverstein *et al.*, 1986; Rothstein *et al.*, 1992; Li *et al.*, 1996), although it has not been directly demonstrated to depend on transporter oxidation. However, recent observations suggest that this is a likely possibility. Thus, glutamate uptake in cultured astrocytes is inhibited by a β-amyloid peptide secondary to oxygen radical formation (Harris *et al.*, 1995, 1996). Moreover, glutamate transporters of transgenic mice with a superoxide dismutase type 1 (SOD-1) mutation linked to familial amyotrophic lateral sclerosis are chemically modified by oxidants like $ONOO^-$ (Rothstein *et al.*, 1996b).

We have shown previously that glutamate transporters are sensitive to a number of biological oxidants, including H_2O_2, the xanthine/xanthine oxidase reaction products and, especially, $ONOO^-$ (Volterra *et al.*, 1994; Trotti *et al.*, 1996). The present data strongly indicate that a major chemical target for these oxidants is the SH-based redox site, as demonstrated also by the interaction of H_2O_2 with redox modulation of glial uptake (Fig. 4). Indeed, in many respects, the action of H_2O_2 appears superimposable on that of DTNB (compare Fig. 3 with Fig. 4). Thus, H_2O_2 reverses DTT potentiation, reducing the uptake current below its native (control) level. In turn, H_2O_2 inhibition is largely (70%) reversed by DTT, which brings the current back above control. Incomplete reversal with DTT could be because H_2O_2, unlike DTNB, drives SH oxidation in part to a level beyond the disulphide, i.e. insensitive to the reducing action of DTT (Radi *et al.*, 1991). Alternatively, H_2O_2 could target additional reactive amino acid residues of transporters (Davies *et al.*, 1987).

The oxidizing power of $ONOO^-$ is greater than that of H_2O_2, and $ONOO^-$ might be formed during pathological brain processes (Beckman, 1994). It inhibits reconstituted glutamate transporters several-fold more potently than H_2O_2 but by an apparently similar mechanism (Trotti *et al.*, 1996). Indeed, preliminary results in our laboratory suggest that $ONOO^-$, like H_2O_2, targets transporter sulphydryls: thus, the compound loses most of its inhibitory potency when applied to transporters pretreated with the thiol modifier NEM (1 mM). The pre-existing effect of NEM (Fig. 1A) reduces the expected effect of $ONOO^-$ (50 μM) by >60%. In addition, $ONOO^-$ could cause nitration of the transporter aromatic amino acid residues (Beckman *et al.*, 1992).

A regulatory redox mechanism based on reactive sulphydryls has been described for a number of proteins, including the NMDA receptors (Aizenman *et al.*, 1989; Sullivan *et al.*, 1994). Oxidation leads to reduced Ca^{2+} permeability through the NMDA channel, with important implications for cell excitability, signalling and neurotoxicity (Aizenman *et al.*, 1990). The existence of similar molecular redox-sensing elements on the glutamate transporters suggests that glutamatergic neurotransmission might have evolved a complex and fine mechanism of integrated regulation of its function in response to physiological or pathological changes of the redox environment.

In conclusion, the redox-sensing property of glutamate transporters demonstrated in this study is important because it suggests that: (i) redox modulation is a possible physiological regulatory mechanism of glutamate uptake; (ii) glutamate transporters are sensitive to oxidation and therefore pathological conditions characterized by increased oxidative stress may be worsened by inhibition of glutamate uptake.

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Abbreviations

CHAPS	3-[(3-cholamido-propyl)dimethylammonio]-1-propane-sulphonate
DTNB	5,5′-dithio-bis(2-nitrobenzoic) acid
DTT	dithiothreitol
EAA	excitatory amino acid
GFAP	glial fibrillary acidic protein
NEM	*N*-ethylmaleimide
pCMB	*p*-chloromercuribenzoic acid
pCMPS	*p*-chloromercuryphenylsulphonic acid
PCSM	reconstitution mixture containing phospholipid, cholate and salt

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