Regulation of Extracellular Glutamate in the Prefrontal Cortex: Focus on the Cystine Glutamate Exchanger and Group I Metabotropic Glutamate Receptors

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ABSTRACT

Microdialysis was used to determine the in vivo processes contributing to extracellular glutamate levels in the prefrontal cortex of rats. Reverse dialysis of a variety of compounds proved unable to decrease basal levels of extracellular glutamate, including Na\(^+\) and Ca\(^{2+}\) channel blockers, cystine/glutamate exchange (X\(_{AC}\)) antagonists, and group I (mGluR1/5) and group II (mGluR2/3) metabotropic glutamate receptor (mGluR) agonists or antagonists. In contrast, extracellular glutamate was elevated by blocking Na\(^+\)-dependent glutamate uptake (X\(_{AC}\)) with DL-threo-β-benzoyloaspartate (TBOA) and stimulating group I mGluRs with (R,S)-3,5-dihydroxy-phenylglycine (DHPG). The accumulation of extracellular glutamate produced by blocking X\(_{AC}\) was completely reversed by inhibiting system X\(_{-}\)c with 4-carboxyphenylglycine (CPG), but not by Na\(^+\) and Ca\(^{2+}\) channel blockers. Because CPG also inhibits group I mGluRs, two additional group I antagonists were examined, LY367385 [(+)2-methyl-4-carboxyphenylglycine] and (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA). Whereas LY367385 also reduced TBOA-induced increases in extracellular glutamate, AIDA did not. In contrast, all three group I antagonists reversed the increase in extracellular glutamate elicited by stimulating mGluR1/5. In vitro evaluation revealed that similar to CPG, LY367385 inhibited X\(_{AC}\) and that stimulating or inhibiting mGluR1/5 did not directly affect \(^{3}H\)glutamate uptake via X\(_{AC}\) or X\(_{AG}\). These experiments reveal that although inhibiting X\(_{-}\)c cannot reduce basal extracellular glutamate in the prefrontal cortex, the accumulation of extracellular glutamate after blockade of X\(_{AG}\) arises predominately from X\(_{-}\)c. The accumulation of glutamate elicited by mGluR1/5 stimulation does not seem to result from modulating X\(_{AG}\), X\(_{c}\), or synaptic glutamate release.

Glutamate is the primary excitatory neurotransmitter in the central nervous system, and alterations in glutamate transmission are implicated in pathologies ranging from neurotoxicity to neuropsychiatric disorders (Choi, 1988; Tsai and Coyle, 2002; Kalivas and Volkow, 2005). Because there is no extracellular enzymatic inactivation of glutamate, cellular uptake is necessary to remove glutamate released into the extracellular space by vesicular and nonvesicular mechanisms (Danbolt, 2001). Glutamate uptake transporters (EAAT1–5) are members of the X\(_{AG}\) family of electrogenic, Na\(^+\)-dependent amino acid transporters. Another glutamate system, referred to as system X\(_{-}\)c, mediates the Na\(^+\)-independent exchange of one intracellular glutamate for one extracellular cystine molecule. Thus, rather than eliminating extracellular glutamate, X\(_{-}\)c is a nonvesicular source of extracellular glutamate (Cho and Bannai, 1990; Sato et al., 1999; Patel et al., 2004), and in vivo microdialysis in the nucleus accumbens revealed that X\(_{-}\)c is responsible for 50 to 70% of basal extracellular glutamate (Baker et al., 2002). Glutamate is continuously being released from a variety of sources to the extracellular fluid. In addition to X\(_{-}\)c, extracellular glutamate is derived from vesicular synaptic and nonvesicular glial release, both of which are calcium-dependent (Danbolt, 2001; Haydon, 2001). Although most studies to date have focused on synaptic release of glutamate from nerve terminals, the primary source of extracellular glutamate measured outside of the synaptic cleft seems to be X\(_{-}\)c. Implicating X\(_{-}\)c, Jabaudon et al. (1999) showed that inhibition of X\(_{AG}\) leads to extracellular accumulation of glutamate from

ABBREVIATIONS: EAAT1–5, excitatory amino acid transporters 1 to 3; X\(_{AG}\), Na\(^+\)-dependent glutamate uptake; X\(_{c}\), cystine-glutamate exchanger; mGluR, metabotropic glutamate receptor; AIDA, (R,S)-1-aminoindan-1,5-dicarboxylic acid; APDC, (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate; CPG, (S)-4-carboxyphenylglycine; DHPG, (R,S)-3,5-dihydroxy-phenylglycine; LY367385, (+)-2-methyl-4-carboxyphenylglycine; MPEP, 2-methyl-6-(phenylethynyl) pyridine; TBOA, dl-threo-β-benzoyloaspartate; AP-5, 2-amino-5-phosphono-pentanoic acid; GVIA, ω-conotoxin GVIA; HCA, homocysteic acid; MVIC, ω-conotoxin MVIIIC; TTX, tetrodotoxin; xCT, catalytic subunit of X\(_{-}\); ANOVA, analysis of variance; NMDA, N-methyl-D-aspartate.
sources that were insensitive to blockade of voltage-dependent Na$^+$ and Ca$^{2+}$ channels or to the administration of toxins that cleave proteins essential for exocytosis.

The in vivo concentration of extracellular glutamate has also been shown to be regulated by group I and group II metabotropic glutamate receptors (mGluR1/5 and mGluR2/3, respectively). In vivo microdialysis studies reveal that stimulation of group I or inhibition of group II mGluRs elevates extracellular glutamate level in various brain regions, including the nucleus accumbens (Swanson et al., 2001; Baker et al., 2002; Xi et al., 2002), parietal cortex (Moroni et al., 1998), and prefrontal cortex (Melendez et al., 2004).

The present study used in vivo microdialysis and in vitro glutamate uptake to determine the contribution of system xC- in regulating extracellular glutamate in the prefrontal cortex. The prefrontal cortex was examined because of its postulated role in drug addiction and schizophrenia and the emerging concept that these neuropsychiatric disorders may involve pathological adaptations in the cellular processes regulating extracellular glutamate levels (Choi, 1988; Tsai and Coyle, 2002; Kalivas and Volkow, 2005).

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250 to 300 g upon arrival, were individually housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility with access to food and water ad libitum. Rooms were set on a 12:12 light cycle, with lights on at 7:00 AM, and all experimentation was conducted during the light period. All protocols were approved by the Institutional Animal Care and Use Committee in compliance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery. Rats were anesthetized with a combination of ketamine (100 mg/kg, i.p.) and xylazine (3 mg/kg, i.p.). Using coordinates derived from the Paxinos and Watson (1998) atlas (in millimeters; +2.7, anterior; +1.1, lateral, –2.0, ventral at a 6° angle from vertical), bilateral microdialysis guide cannulae were implanted above the prefrontal cortex. Guide cannulae were secured to the skull using four skull screws (Small Parts, Roanoke, VA) and dental acrylic. After surgery, rats were permitted at least 5 days to recover before testing.

Microdialysis. Microdialysis probes were constructed with both the inlet and outlet tubing consisting of fused silica (Baker et al., 2002). The active region of the dialysis membrane was 3 mm in length and –0.22 mm in diameter. The night before the dialysis experiment, a probe was inserted unilaterally through the guide cannulae into the prefrontal cortex. The next morning, dialysis buffer (5 mM glucose, 5 mM KCl, 140 mM NaCl, 1.4 mM CaCl$_2$, and 1.2 mM MgCl$_2$, and 0.5% phosphate-buffered saline was added to pH 7.4) was advanced through the probe at a rate of 3 μl/min via syringe pump (BAS Bioanalytical Systems, West Lafayette, IN). Two hours later, baseline samples were collected. The standard protocol used for microdialysis experiments involved the collection of five 20-min baseline samples, followed by three additional 20-min samples for each concentration of a given drug. Thus, multiple doses of each compound were administered in each rat. Liquid switches were used to minimize the pressure fluctuations while changing dialysis buffers with varying drug concentrations.

Dosage ranges of the various drugs were based upon the relative EC$_{50}$ or IC$_{50}$ values for binding to the respective receptors (Shimamoto et al., 1998; Schoepf et al., 1999; Gochenauer and Robinson, 2001) or concentrations shown effective in previous microdialysis studies (Swanson et al., 2001; Baker et al., 2002). All drugs were purchased from Tocris Cookson Inc. (Ellisville, MO) and were freshly prepared on the day of the experiment. (R,S)-1-Aminoundan-1,5-dicarboxylic acid (AIDA), (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC), (S)-4-carboxyphenylglycine (CPG), (R,S)-3,5-dihydroxyphenylglycine (DHPG), LY367385, 2-methyl-6-(phenylethyl)pyridine (MPEP), and tetrathio-benzoxysapartate (TBOA) were initially dissolved in 0.1 N NaOH and neutralized with 0.1 N HCl. Working concentrations were then made by diluting with filtered dialysis buffer. 2-Amino-5-positononopentanoic acid (AP-5), diltiazem, ω-conotoxin GVIA (GVIA), homocysteic acid (HCA), ω-conotoxin MVIIIC (MVIIIC), EGTA, and tetrodotoxin (TTX) were dissolved in filtered dialysis buffer.

Quantification of Glutamate. Microdialysis samples were collected into vials containing 10 μl of 0.05 M HCl. The concentration of glutamate in the dialysis samples was determined using high-performance liquid chromatography with fluorometric detection. Precolumn derivitization of glutamate with O-phthalaldehyde was performed using a Gilson 231 XL autosampler (Gilson Medical Electronics, Middleton, WI). The mobile phase consisted of 11% acetonitrile (v/v), 100 mM Na$_2$PO$_4$, and 0.1 mM EDTA, pH 6.04. Glutamate was separated using a reversed-phase column (3 μm; 100 × 4.2 mm; BAS Bioanalytical Systems) and was detected using a Shimadzu (Columbia, MD) 10RF-A fluorescence detector with an excitation wavelength of 520 nm and an emission wavelength of 400 nm. The concentration of glutamate in the dialysis samples was quantified by comparing peak heights from samples and external standards.

Histology. After completion of the microdialysis experiments, rats were deeply anesthetized using CO$_2$ inhalation. The brains were removed and stored in 1% formalin for at least 1 week before sectioning. The tissue was then blocked, and coronal sections (100 μM) were cut and stained with cresyl violet to verify probe placements. Only animals with probes located in the prefrontal cortex were included in the data analysis.

L-[H]$^3$Glutamate Uptake Assay. Rats were decapitated and the prefrontal cortex was rapidly dissected and cut into 350 × 350-μm prism-shaped slices using a McIlwain tissue chopper (Vibratome, St. Louis, MO). The slices were washed for 30 min at 37°C in oxygenated Krebs-Ringer solution phosphate buffer (140 mM NaCl, 1.3 mM CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 5 mM HEPES, 10 mM glucose, and 1 mM MgCl$_2$) with a final pH of 7.4. Glutamate uptake measurements were initiated by adding L-[H]$^3$glutamate (250 nM, 51 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) in the presence of 10 μM unlabeled L-[H]$^3$glutamate in a final volume of 250 μl of oxygenated buffer. After incubation at 37°C for 15 min, the uptake was terminated by washing the slices in ice-cold nonradioactive choline-containing buffer. Na$^+$-independent uptake was measured by replacing NaCl with equal concentrations of choline chloride. Under these conditions, L-[H]$^3$glutamate uptake was shown to be Na$^+$-, time-, temperature-, and concentration-dependent (data not shown). Slices were then solubilized using 1% SDS, and the level of radioactivity was determined using a liquid scintillation counter. Protein content in the slices was measured using the Bradford assay.

Immunoblotting and Immunochemistry. Dissected nucleus accumbens and prefrontal cortex tissues were homogenized with a hand-held tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% SDS, 50 μM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin; pH 7.2), subjected to low-speed centrifugation (2000g, to remove insoluble material), and frozen at 80°C. Protein determinations were performed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Samples (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis using a Mini gel apparatus (6%; Bio-Rad), transferred via semidyram apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (one gel per protein per brain region). The rabbit anti-rat antibody against xCT was generated at the Medical University of South Carolina and used at a dilution of 1:500. Characterization of this antibody is described in detail previously (Szumlinski et al.,...
For immunocytochemistry, brains were fixed with 4% paraformaldehyde via intracardiac perfusion and stored overnight in 2% paraformaldehyde. Coronal slices of prefrontal cortex (30 μm in thickness) were made with a Vibratome and incubated overnight at room temperature in xCT antibody (1:2500). The sections were washed three times in phosphate-buffered saline and sequentially incubated with biotinylated anti-rabbit secondary antibody and preformed avidin-biotinylated enzyme complex according to product guidelines (Vectastain; Vector Laboratories, Burlingame, CA). Tissues were then stained with diaminobenzidine and examined with light microscopy.

Statistical Analysis. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular glutamate levels. A two-way ANOVA with repeated measures over time was used to compare glutamate between treatments and within treatments over time. Post hoc comparisons were made using Fisher’s least significant difference test. Differences in the effects drugs on [3H]glutamate uptake were analyzed by one-way ANOVA.

Results

Basal Level of Extracellular Glutamate Levels in the Prefrontal Cortex. Figure 1A and Table 1 summarize the results of the microdialysis experiments in which attempts were made to alter the basal level of extracellular glutamate in the prefrontal cortex using various concentrations of drugs that target specific mechanisms postulated to contribute to glutamate release or elimination. Using a relatively high concentration of drugs, various Ca2+ channel blockers failed to alter basal extracellular glutamate levels, including ω-conotoxin MVIIC (N-, P-, and Q-type), ω-conotoxin GVIA (N-type), diltiazem (L-type), or EGTA (chelator of extracellular Ca2+). Similarly, the voltage-dependent Na+ channel blocker TTX or the NMDA-gated Ca2+ channel blocker AP-5 was without effect.

Previous studies using in vivo microdialysis have shown
that the inhibitors of x_c, CPG, or HCA, and the mGluR2/3 agonist APDC decreased basal levels of extracellular glutamate in the nucleus accumbens (Baker et al., 2002; Xi et al., 2002). In the present study, 0.5 to 500 μM CPG failed to alter the extracellular levels of glutamate in the prefrontal cortex. Surprisingly, 100 μM HCA produced a significant increase in extracellular glutamate (Fig. 1A). However, HCA is also an NMDA agonist, and Fig. 1D shows that the HCA-induced increase in glutamate was reversed by co-perfusion with the NMDA agonist AP-5 (500 μM). Also, the reduction observed in glutamate by stimulating mGluR2/3 in the accumbens (Baker et al., 2002) was not observed in the prefrontal cortex (Fig. 1A) after application of 0.5 to 500 μM APDC.

In contrast to the distinction between the accumbens and prefrontal cortex regarding compounds that decrease basal levels of extracellular glutamate, similar to previous reports in the accumbens (Swanson et al., 2001; Baker et al., 2002; Xi et al., 2002), reverse dialysis of compounds that stimulate the accumulation of extracellular glutamate was effective in prefrontal cortex. First, blockade of X_AG by TBOA elevated extracellular glutamate in the prefrontal cortex to nearly 375% of baseline (Fig. 1, A and B). Also, the group I agonist DHPG (5–500 μM) was also without effect on Na^+-independent [3H]glutamate uptake (Fig. 3A). Consistent with the structural similarity between CPG and LY367385 (Fig. 3D), 5 to 500 μM TBOA, whereas Na^+-independent [3H]glutamate uptake was blocked by 5 to 500 μM CPG. Similarly, application of 250 to 5000 μM cystine dose-dependently inhibited system x_c (maximal inhibition 23% of baseline; data not shown). CPG (500 μM) did not alter Na^+-independent [3H]glutamate uptake, and 500 μM TBOA did not affect Na^+-independent [3H]glutamate uptake (Fig. 4A). Consistent with the structural similarity between CPG and LY367385 (Fig. 2D), 5 to 500 μM LY367385 caused a dose-dependent reduction in Na^+-independent [3H]glutamate uptake similar to CPG (Fig. 4A), whereas 50 to 1500 μM AIDA was without effect (Fig. 4B). The mGluR1/5 antagonist MPEP (1–100 μM) was also without effect on Na^+-independent [3H]glutamate uptake (Fig. 4B). To directly verify that the effect of CPG on system x_c and X_AG did not result from blocking mGluR1/5, 500 μM DHPG was co-applied with 500 μM CPG or 500 μM TBOA. As shown in Fig. 4C, DHPG did not reverse the CPG-induced decrease in Na^+-independent [3H]glutamate uptake (Fig. 4C). DHPG (5–500 μM) alone did not alter Na^+-independent and -dependent [3H]glutamate uptake.

**Identification of x_c in the Prefrontal Cortex.** Using an antibody against the catalytic subunit of x_c (xCT; Szumlinski et al., 2004), Fig. 5 shows immunohistochemical and immunoblotting evidence that x_c is present in the prefrontal cortex. Figure 5A shows that most, if not all, cells in the prefrontal cortex express xCT, and the higher magnification micrograph in Fig. 5B shows that some of the immunoreactivity seems to be present in clusters that are in or adjacent to the cell membrane. Figure 5C shows immunoblot com-
paring the level of xCT in tissue dissected from the prefrontal cortex or nucleus accumbens. Similar amounts of xCT were found in both regions.

**Histology.** Figure 6 verifies the location of dialysis probes used in this study. The majority of probes spanned the prefrontal cortex with a portion of the active membrane region in either the anterior cingulate or infralimbic cortex (Paxinos and Watson, 1998).

**Discussion**

The present study demonstrates that in vivo pharmacological blockade of Na\(^+/\)H\(^+\)-dependent glutamate transporters increases the basal concentration of extracellular glutamate in the prefrontal cortex and that the accumulation of glutamate does not result from synaptic or Ca\(^{2+}\)-dependent glutamate release. Rather, the accumulation of extracellular glutamate resulted from the release of glutamate by system x\(_{c}^-\). However, the basal level of extracellular glutamate was not affected by voltage-dependent channel blockers or by inhibitors of x\(_{c}^-\).

**Basal Levels of Glutamate.** Akin to previous in vivo studies examining both cortical and subcortical brain regions, the basal extracellular level of glutamate was not affected by blocking various voltage-dependent ion channels associated with vesicular glutamate release (Timmerman and Westerink, 1997; Del Arco et al., 2003). However, the inability to reduce extracellular glutamate in the prefrontal cortex with the x\(_{c}^-\) antagonist CPG in the prefrontal cortex is in contrast with the effect of CPG in the nucleus accumbens (Baker et al., 2002). The cellular basis of the difference between the two brain regions is unclear given the presence of substantial immunoreactive xCT (Shih and Murphy, 2001) in the prefrontal cortex. However, the basal extracellular concentration of glutamate determined by no net flux dialysis is about 2-fold greater in the nucleus accumbens than in the prefrontal cortex, and CPG reduces the level of basal glutamate in the accumbens to approximately the level of basal glutamate in the prefrontal cortex (Baker et al., 2002, 2003; Xi et al., 2002). Thus, in either structure, inhibition of x\(_{c}^-\) did not reduce glutamate levels below approximately 2 \(\mu\)M, and...
the remainder of glutamate was unaffected by inhibitors of synaptic glutamate release. One possible contribution to the 2 μM extracellular glutamate that is unaffected by Xc- antagonists or voltage-dependent ion channel blockers is from decreasing efficacy of XAG to eliminate glutamate. Although estimates of the K_m of glutamate uptake vary greatly between preparations, most typical values range between 5 and 30 μM (Danbolt, 2001). Moreover, in some studies the rate of glutamate unbinding from EAAT1 was found to exceed the rate of translocation into the cell, indicating that as the extracellular concentration is reduced, glutamate may be maintained in part by unbinding from transporters (Wadiche and Kavanaugh, 1998). Another possible contributor is the vesicular glutamate transporter that has been proposed to leak glutamate into the extracellular space when present in the plasma membrane or other relatively poorly characterized transmembrane diffusion processes postulated to permit diffusion of glutamate into the extracellular space (Danbolt, 2001). Of particular interest for in vivo microdialysis estimates of extracellular glutamate is the release of glutamate produced by osmotic challenge (Kimelberg et al., 1990; Strange et al., 1996). Although the perfusion buffer used in dialysis studies is designed to be isosmotic, unavoidable perturbations of the extracellular space by the probe may result in cell swelling and subsequent release of glutamate.

Another interesting distinction between the accumbens and prefrontal cortex was the fact that over the dosage range used, HCA in the accumbens acts predominately as an Xc- antagonist to reduce basal levels of glutamate (Baker et al., 2003), but in the prefrontal cortex HCA elevated glutamate. It was possible that this elevation resulted from HCA acting as a false cystine substrate in the prefrontal cortex, thereby stimulating heteroexchange with intracellular glutamate (Patel et al., 2004). However, the elevation in glutamate by HCA was abolished by coperfusion with the NMDA blocker AP-5, arguing that the increase resulted entirely from the known efficacy of HCA as an agonist at NMDA receptors (Lehmann et al., 1988).

The preferential effect of HCA on NMDA receptors relative to Xc- may result from the relative abundance of NMDA

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**Fig. 3.** DHPG-induced elevation of extracellular glutamate levels is inhibited by CPG, LY367385, and AIDA. A, significant increase [F_1.9 = 18.5; p < 0.05] in extracellular glutamate induced by 50 μM DHPG (n = 4) was not inhibited by coapplication of 1 μM TTX (n = 5) and 10 μM MVIIIC (n = 5). However, the DHPG-induced increase in extracellular glutamate was significantly inhibited by coapplication of 50 μM CPG (n = 5), 50 μM LY367385 (n = 5), and 500 μM AIDA (F_1.9 < 3.1; p > 0.1). Glutamate levels are expressed as percentage ± S.E.M. of basal pretreatment levels. B to D illustrate the time course used to sample extracellular glutamate in the prefrontal cortex before and after reverse microdialysis. The black lines indicate the beginning of local perfusion (i.e., reverse dialysis) of the respective drugs. B, two-way ANOVA with repeated measures over time revealed that the excitatory effect of 50 μM DHPG (n = 4) was competitively reversed by coapplication of 0.5 to 50 μM CPG (n = 5; time × group interaction) (F_17,119 = 2.5; p < 0.05). C, excitatory effect of 50 μM DHPG (n = 4) was competitively reversed by coapplication of 0.5 to 50 μM LY367385 (n = 4; time × group interaction (F_17,102 = 9.7; p > 0.05). D, excitatory effect of 50 μM DHPG (n = 4) was competitively reversed by coapplication of 150 to 500 μM AIDA (n = 5; time × group interaction) (F_14,98 = 11.8; p < 0.05). *p < 0.05 compared with baseline; +, p < 0.05 compared with DHPG alone.
receptors in the cortex compared with the nucleus accumbens (Monaghan and Cotman, 1986), or perhaps different phosphorylation states of xCT may result in reduced capacity of HCA to inhibit $\chi^-$ (Gochenauer and Robinson, 2001; Baker et al., 2003; Tang and Kalivas, 2003).

**Nonvesicular Origin of Extracellular Glutamate Accumulated by Blocking XAG.** It was previously shown using in vitro electrophysiological estimates of extracellular glutamate that the accumulation of glutamate after blockade of XAG with TBOA was not derived from synaptic release since it was unaffected by blockade of $\text{Na}^+$ or $\text{Ca}^{2+}$ channels or by the administration of peptide toxins that cleave proteins required for vesicular release (Jabaudon et al., 1999). Consistent with this elegant in vitro study, the accumulation of extracellular glutamate in vivo was unaffected by compounds that inhibit synaptic glutamate release. In contrast, the inhibition of $\chi^-$ by CPG completely reversed the elevation in extracellular glutamate produced by TBOA. In addition to inhibiting $\chi^-$, CPG blocks mGluR1/5 (Ye et al., 1999), and CPG also reversed the increase in extracellular glutamate produced by reverse dialysis of the mGluR1/5 agonist DHPG. However, a selective effect on $\chi^-$ by CPG was revealed by the fact that another mGluR1/5 antagonist AIDA inhibited the effect of DHPG but did not reduce the increase in glutamate by TBOA. The lack of effect by AIDA to inhibit $\chi^-$ has been further verified in vitro using $\text{Na}^+$-independent $[^3\text{H}]$glutamate (Fig. 4) or $[^35\text{S}]$cystine uptake in tissue slices (Baker et al., 2002).

Although the present experiments demonstrated that the effect of CPG on TBOA-induced accumulation of extracellular glutamate in vivo resulted from the inhibition of $\chi^-$, it is important to note that TBOA-induced accumulation of glutamate may not reflect a physiological function of $\chi^-$. More-
over, the microdialysis experiments could not exclude a role for $x_c^-$ in the elevation of glutamate elicited by stimulating mGluR1/5. The accumulation of glutamate by DHPG was apparently nonsynaptic since it was unaffected by blocking voltage-dependent Na$^+$ or Ca$^{2+}$ channels. However, since the effective $x_c^-$ antagonists CPG and LY367385 also are mGluR1/5 antagonists, it remained possible that stimulating mGluR1/5 with DHPG could have altered $x_c^-$ via mGluR1/5-mediated cell signaling. For example, mGluR1/5 is positively coupled to phospholipase C intracellular signaling via calcium-dependent protein kinase in inositol triphosphate, which have been proposed previously to underlie the ability of DHPG to release glutamate (Cochilla and Alford, 1998; Schwartz and Alford, 2000; Swanson et al., 2001). Moreover, stimulating protein kinase C inhibits $^{35}$S cystine uptake in astrocytes (Tang and Kalivas, 2003). However, arguing against mGluR1/5 regulation of $x_c^-$, DHPG did not affect Na$^+$-independent $^{3}$H glutamate uptake in prefrontal cortical slices (Fig. 4C).

**LY367385 Is a Novel Inhibitor of System $x_c^-$**

As has been the case with Na$^+$-dependent transporters, the most potent inhibitors of $x_c^-$ identified to date are conformationally constrained analogs (Patel et al., 2004). By restricting bond rotations, the functional groups on the molecule can potentially be locked in a configuration that mimics the endogenous substrate. This strategy, which is commonly accomplished by introducing ring systems into the carbon backbone, often results in both increased potency and specificity (Chamberlin et al., 1998). CPG, a conformationally restricted compound, has been shown to be one of the most potent and selective inhibitors of system $x_c^-$ (Gochenauer and Robinson, 2001; Patel et al., 2004). Unlike the group I antagonists AIDA and MPEP, LY367385 is a conformationally restricted compound and phenylglycine derivative of CPG (Fig. 2D). In the present study, LY367385, but not AIDA and MPEP, dose-dependently reduced system $x_c^-$. Furthermore, LY367385 was effective in reducing the TBOA-induced accumulation of extracellular glutamate in the prefrontal cortex as measured by in vivo microdialysis. To our knowledge, this is the first study showing the in vivo and in vitro effects of LY367385 on system $x_c^-$ activity.

**Summary.** The majority of extracellular glutamate measured in the prefrontal cortex by in vivo microdialysis originates from unidentified nonvesicular, nonsynaptic sources. The accumulation of extracellular glutamate produced by inhibiting $X_{AG}$ was also shown to nonsynaptic in origin, but system $x_c^-$ was identified as the source of accumulated glutamate.

**References**


Murphy TH, Miyamoto M, Sastre A, Schnaar RL, and Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 3:1547–1558.


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