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Effects of increased extracellular glutamate levels on the local field potential in the brain of anaesthetized rats

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1 It is generally considered that glutamate-mediated transmission can be altered from a physiological to neurotoxic action when extracellular glutamate levels become excessive subsequent to impaired uptake and/or excessive release. However, high extracellular glutamate does not consistently correlate with neuronal dysfunction and death *in vivo*. The purpose of this study was to examine *in situ* the local depolarizations, as indicated by negative shifts of the extracellular field (d.c.) potential, produced by local inhibition of high-affinity glutamate uptake, with or without co-application of exogenous glutamate, in three brain regions of anaesthetized rats.

2 Microdialysis probes incorporating an electrode were used to apply exogenous glutamate and/or its uptake inhibitor L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC), and to monitor the resulting changes in extracellular glutamate and d.c. potential at the sites of application within the cortex, striatum and hippocampus.

3 Perfusion of 1 to 10 mM L-*trans*-PDC markedly and concentration-dependently increased extracellular glutamate levels (by up to 1700% of basal level in the parietal cortex). Despite their large magnitude, glutamate changes were associated with minor negative shifts of the d.c. potential (<2 mV), which were not suppressed by the *N*-methyl-D-aspartate (NMDA)-channel blocker, dizocilpine (MK-801, 2 mg kg^{-1}, i.v.), or the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainate-receptor antagonist, 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX, 30 mg kg^{-1}, i.p.). L-*trans*-PDC had virtually identical concentration-dependent effects on dialysate glutamate in the hippocampus and striatum, but those induced in the cortex were around 40% larger ($P<0.002$). In contrast, the associated depolarizations were around twice as large in the striatum and cortex as in the hippocampus ($P<0.002$). Finally, co-application of L-*trans*-PDC did not enhance the d.c. potential changes evoked by perfusion of 5 or 20 mM glutamate.

4 As the neurotoxic potency of glutamate agonists is considered to be linked to excessive opening of glutamate-operated ion channels, these results challenge the notion that high extracellular glutamate levels may be the key to excitotoxicity in neurological disorders. In particular, they do not support the hypothesis that high extracellular glutamate causes the sudden negative shifts of the d.c. potential associated with ischaemia (i.e. anoxic depolarization), traumatic brain injury and spreading depression. Impaired uptake and excessive release of glutamate may well lead to excitotoxicity, but only at the synaptic level, not by spreading through the interstitial fluid.

Keywords: Glutamate; excitotoxicity; glutamate-uptake; microdialysis; L-*trans*-pyrrolidine-2,4-dicarboxylate; glutamate receptors

Introduction

Neuronal death subsequent to excessive stimulation (excitotoxicity) may be involved in a number of neurological disorders, including stroke, epilepsy and neurodegeneration (Meldrum, 1994). With regard to stroke, four lines of evidence have led to the concept that excitatory neurotransmitter glutamate becomes neurotoxic because extracellular levels become excessive when its uptake by cells and synapses is impaired and/or its presynaptic release enhanced. Thus, glutamate is toxic to cultured neurones (Choi, 1987), glutamate antagonists are neuroprotective (McCulloch *et al*., 1993), extracellular glutamate increases during ischaemia (Obrenovitch *et al*., 1993b) and glutamate-uptake of isolated glial cells is altered by ionic changes similar to those occurring in ischaemia (Szatkowski & Attwell, 1994; see, however, Zerangue & Kavanaugh, 1996). However, high extracellular glutamate does not consistently correlate with, nor necessarily produce, neuronal dysfunction and death *in vivo* (Obrenovitch & Urenjak, 1997). For example, the ischaemic penumbra (Obrenovitch, 1995) is selectively protected by antagonists of ionotropic glutamate receptors (McCulloch *et al*., 1993), but there is no sustained increase of the extracellular concentration of glutamate in this region (Obrenovitch *et al*., 1993b; Obrenovitch, 1996).

In previous experiments, intracerebral application of glutamate induced a concentration-dependent depolarization, as indicated by local negative shifts of the extracellular field (d.c.) potential, but glutamate was 200 to 400 fold less potent than its agonists, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) or kainate (Obrenovitch *et al*., 1994). One explanation for the low depolarizing potency of glutamate in these conditions may be its effective removal, from the extracellular space into the cells, as it is applied to the brain (Garthwaite *et al*., 1992). The purpose of this study was to examine, *in situ* and in several brain structures, whether changes in the d.c. potential indicative of depolarization occur when extracellular glutamate is increased by inhibition of its high-affinity uptake and/or application of exogenous glutamate.

Microdialysis probes incorporating a microelectrode (Obrenovitch *et al*., 1993a; 1994) were used in anaesthetized rats to determine how increasing concentrations of the selective glutamate uptake inhibitor, L-*trans*-pyrrolidine-2,4-dicarboxylate (L-*trans*-PDC; Bridges *et al*., 1991) alter (i) the basal d.c. potential at the site of application and (ii) depolarizations evoked by co-application of exogenous glutamate. Dialysate

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glutamate levels were continuously monitored by enzyme-induced fluorescence.

Some of these results have been presented to the British Pharmacological Society (Urenjak *et al*., 1995; 1996).

Methods

Animal preparation and intracerebral microdialysis

Adult male Sprague-Dawley rats (250–350 g; Bantin & Kingman, Grimson, Hull, U.K.) were used with food and water available *ad libitum*. All animal procedures were in strict accordance with the British Home Office guidelines, and specifically licensed under the Animals (Scientific Procedures) Act 1986. Anaesthesia was induced and maintained during surgery with halothane (2.5% and 1.5–2.0%, respectively) in O_2:N_2O (1:1), with the animal breathing spontaneously. To minimize any possible interference of halothane anaesthesia with the processes under study (Carla & Moroni, 1992; Martin *et al*., 1995), once the surgical procedure had been completed, the depth of anaesthesia was carefully controlled by monitoring EEG and arterial blood pressure, and the concentration of halothane in the breathing mixture kept to a minimum (0.8 to 1.2%). A femoral artery was catheterized for continuous monitoring of mean arterial pressure. A femoral vein was cannulated for administration of drugs and rapid injection of 1 ml air to terminate the experimental procedure by cardiac arrest. Body temperature was kept at 37–38°C throughout.

Identical microdialysis probes incorporating an electrode (Obrenovitch *et al*., 1993a) (AN69 Hospal Medical fibre, 0.3 mm o.d. and 2 mm length) (ME-H2, Applied Neuroscience, London) were implanted in the parietal cortex (2 mm lateral, 1.3 mm posterior to bregma, 2 mm from the dural surface), striatum (3 mm lateral, 0.8 anterior to bregma, 6 mm deep) or hippocampus (2.8 lateral, 5.2 posterior to bregma, 4 mm deep; Paxinos & Watson, 1986). Unless otherwise stated, microdialysis probes were perfused with an artificial cerebrospinal fluid (aCSF; composition in mM: NaCl 125, KCl 2.5, $MgCl_2$ 1.18, $CaCl_2$ 1.26; unbuffered, pH 7.3 adjusted with 1 M NaOH) at 1 μl min^{-1} with a syringe pump (CMA/100, CMA/Microdialysis, Stockholm).

Recording of EEG and extracellular d.c. potential

These electrophysiological parameters were derived from the potential between the electrode built into the probe and a chlorided silver reference electrode placed under the scalp (Obrenovitch *et al*., 1993a; 1994). The signal was first amplified ($\times 10$) with a high-impedance input pre-amplifier (NL834, Neurolog System, Digitimer Ltd., Welwyn Garden City, U.K.). The alternating current component in the 1–30 Hz window, amplified 6,000 to 8,000 times, provided the EEG, and the d.c. component, the d.c. potential. An application programme written in ASYST (Keithley, Reading, U.K.) allowed these parameters to be continuously acquired, displayed, and stored on an IBM-compatible PC equipped with an analogue/digital converter (DAS8, Metrabyte Corp., Taunton, MA).

Monitoring changes in dialysate glutamate

Glutamate concentration in the dialysate was determined by on-line fluorometric detection of NADH resulting from the reaction of glutamate and NAD^+ catalyzed by glutamate dehydrogenase (Obrenovitch *et al*., 1990). Briefly, a peristaltic pump (Minipuls 3, Gilson France, Villiers Le Bel, France; 10 μl min^{-1} flow rate) mixed the enzymatic reagent with either a standard solution of L-glutamate (20 μM) or with the brain dialysate as it emerged from the implanted microdialysis probe. The enzymatic reaction developed in a polyethylene tubing (0.4 mm i.d., Portex Ltd., Hythe, U.K.) through which the reagent-dialysate solution flowed to the fluorescence spectrophotometer (Perkin Elmer LC-240, Beaconsfield, U.K.). The dead volume of this tubing permitted a reaction time of approximately 10 min. Changes in NADH were detected with a 4 μl flow cell, at 345 nm excitation and 455 nm emission.

Experimental procedures

After 2 h of stabilization, rats were subjected to one of the following procedures described below.

Effects of inhibition of glutamate uptake on dialysate glutamate and basal d.c. potential

Increasing concentrations of L-*trans*-PDC (1 to 10 mM) were each perfused for 20 min through a microdialysis electrode implanted in the cortex, striatum or hippocampus. Each drug application was followed by 15 min of perfusion with aCSF alone (Figure 1a). Changes in dialysate glutamate and d.c. potential were monitored continuously as described above.

*Origins of depolarizations induced by L-*trans*-PDC*

We examined whether ionotropic glutamate receptors contribute to L-*trans*-PDC-induced depolarizations, by using systemic administration of dizocilpine (MK-801) and 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX) to block NMDA and AMPA/kainate receptors, respectively. Microdialysis electrodes were implanted in the striatum, and 10 mM L-*trans*-PDC was perfused through the probe during separate periods of 20 min. Each intracerebral drug application was followed by 30 or 45 min of perfusion with aCSF alone (Figure 2). In a control group, animals were not treated with any inhibitor of ionotropic glutamate receptors. In a second group, animals received MK-801 (2 mg kg^{-1}, i.v.) 15 min after the first L-*trans*-PDC application, and NBQX (30 mg kg^{-1}, i.p.; Chizh *et al*., 1994) 15 min after the second. This order was reversed in a third group. This experimental design allowed us to examine the effect of MK-801 and NBQX either alone or in combination.

*Effect of L-*trans*-PDC on glutamate-evoked depolarizations*

Microdialysis electrodes were implanted in the striatum of rats, and paired 5 and 20 mM glutamate stimuli perfused for 2 min, each followed by 20 min of recovery. This sequence was repeated 4 times (Figure 3a,b). In a control group, normal aCSF was perfused throughout, except during the glutamate challenges. In the L-*trans*-PDC group, 1 mM of this selective glutamate-uptake inhibitor was perfused starting 10 min after the end of the first 20 mM glutamate stimulus, and replaced by 5 mM L-*trans*-PDC 10 min after the second 20 mM glutamate stimulus. Application of L-*trans*-PDC was pursued during the 2nd and 3rd paired glutamate challenges.

Switching of solutions perfused through the microdialysis probe was performed with a liquid switch with no dead volume (CMA/110, CMA/Microdialysis). Switching solutions in itself did not produce any shifts of the d.c. signal, nor any interference with glutamate monitoring by enzyme-induced fluorescence.

Drugs

The following drugs were used: L-*trans*-PDC and NBQX (Tocris Cookson, Bristol, U.K.); glutamic acid (Sigma Chemicals, Poole, UK); MK-801 (Research Biochemical Inc. Natick, MA). Other chemicals were of analytical grade (Merck/BDH, Poole, U.K.). All solutions to be perfused through the microdialysis probe had their pH carefully adjusted to 7.3 with 1 M NaOH, but were left unbuffered to avoid possible interference with the local acid-base homeostasis. MK-801 was dissolved in sterile saline (0.9% w/v NaCl). NBQX was dissolved in sterile distilled water containing around 35 mM

NaOH and the solution was subsequently neutralized with 1 M HCl to pH 8.

Data presentation and statistical analysis

The polarity of the d.c. potential in Figures 1a, 2 and 3a,b (i.e. representative d.c. potential traces) was defined so that negative shifts produce upwards voltage deflection. In Figure 3a and b, traces were aligned by setting the 2 min preceding glutamate application to 0 mV. All values are presented as mean $\pm$ s.e.mean. In Figure 1a, glutamate and d.c. data were shifted to account for the delay necessary for glutamate analysis (i.e. around 10 min).

Glutamate and d.c. potential means at the same dose of L-*trans*-PDC for different areas of the brain (procedure (i); Figure 1b,c) were compared by analysis of variance. Depolarizations induced by 10 mM L-*trans*-PDC in animals treated with glutamate-receptor antagonists were compared to those of the control group as follows: for each animal, the magnitude of the 2nd and 3rd depolarization (areas under the curve, see Figure 2) was expressed as % of the 1st, because the latter was consistently higher than the subsequent. These normalized values were then compared between groups by a nonparametric test (Mann-Whitney test). The means of the d.c. shifts produced by repeated applications of glutamate (1 and 5 mM) in the control group (Figure 3) were compared by analysis of variance. As this test could not be used for comparison between control and L-*trans*-PDC groups because of one missing value in the latter, differences between the two groups were assessed by Student's *t* test.

P values not greater than 0.05 were deemed to be statistically significant.

Results

Effects of glutamate-uptake inhibition on dialysate glutamate and basal d.c. potential

Basal levels of dialysate glutamate were 1.19 ± 0.13 μM (cortex, $n = 12$), 0.63 ± 0.05 μM (striatum, $n = 6$) and 0.58 ± 0.05 μM (hippocampus, $n = 6$). Perfusion of 1 to 10 mM L-*trans*-PDC markedly and concentration-dependently increased extracellular glutamate levels (Figure 1a,b). Glutamate levels were significantly higher in the cortex compared to the striatum and hippocampus at 2.5, 5.0 and 10.0 mM L-*trans*-PDC. In con-

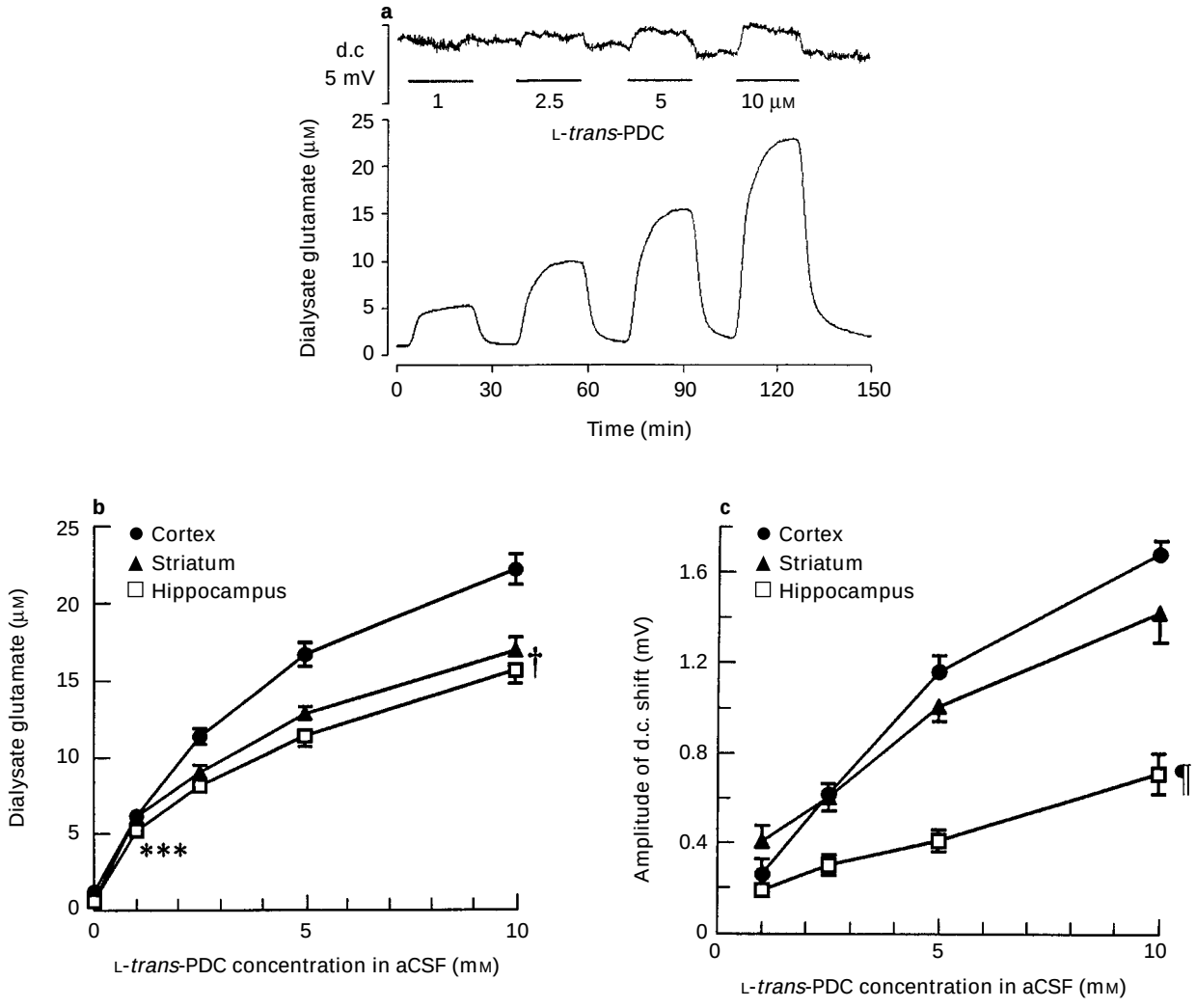

Figure 1 Effects of glutamate-uptake inhibition by L-*trans*-PDC on extracellular concentrations of glutamate and basal d.c. potential. (a) Representative changes in dialysate glutamate (bottom trace) and d.c. potential (top trace) produced in the parietal cortex of anesthetized rats by perfusion of increasing concentrations of L-*trans*-PDC (solid horizontal bars). (b and c) Concentration-dependent increases in dialysate glutamate concentration and negative shift of the d.c. potential produced by L-*trans*-PDC in the cortex, striatum and hippocampus. *** $P < 0.001$ (Student's paired *t* test), for the 3 regions studied, compared to the respective basal glutamate levels. †Comparisons of glutamate levels at 10 mM L-*trans*-PDC in the three regions studied: overall $P < 0.001$ (analysis of variance, $F = 12.88$, d.f. 2, 21); striatum vs cortex, $P < 0.001$ (*t* test); hippocampus vs cortex, $P = 0.002$ (*t* test). ¶Comparisons of the d.c. shift at 10 mM L-*trans*-PDC in the three regions studied: overall $P < 0.001$ (analysis of variance, $F = 33.46$, d.f. 2, 21); striatum vs cortex, $P < 0.04$ (*t* test); hippocampus vs cortex, $P < 0.001$ (*t* test).

trast, striatal and hippocampal glutamate data were not significantly different from one another at any L-*trans*-PDC concentration.

The marked increases in extracellular glutamate produced by L-*trans*-PDC were associated with minor negative shifts of d.c. potential (Figure 1). For example, in the parietal cortex, the increase of dialysate glutamate by 10 mM L-*trans*-PDC (1700% of basal level) was associated with a depolarization of approximately 1.7 mV. As already mentioned, L-*trans*-PDC had virtually identical concentration-dependent effects on dialysate glutamate in the hippocampus and striatum (Figure 1b) but the associated depolarizations were around twice as large in the striatum (Figure 1c).

Effects of glutamate receptor blockade on depolarizations induced by L-trans-*PDC*

Repeated applications of 10 mM L-*trans*-PDC into the striatum produced a consistent pattern of responses, with the first depolarization rising initially to a much higher level, before gradually decreasing as L-*trans*-PDC was still applied (Figure 2). This feature contrasted with the corresponding, progressive increase of dialysate glutamate levels (Figure 1a). In the control group, the areas below the 3 consecutive responses were 44.9 ± 2.4, 35.6 ± 3.4 and 31.6 ± 3.4 mV min (mean $\pm$ s.e.mean, $n = 6$) and the first depolarization was significantly larger than the subsequent ($P < 0.005$, Student's paired t test). The magnitude of the 2nd and 3rd depolarization relative to the first (i.e. approximately 78 and 70%, respectively) was not significantly altered by MK-801 and/or NBQX (Table 1).

Effect of L-trans-*PDC on glutamate-evoked depolarizations*

During basal conditions, application of 5 or 20 mM glutamate to the rat striatum for 2 min evoked depolarizations of 1.28 ± 0.06 and 4.02 ± 0.13 mV, respectively (mean $\pm$ s.e. mean, $n = 14$). In control experiments, responses evoked by 20 mM glutamate did not change significantly with time ($P = 0.29$; analysis of variance, $F = 1.36$, d.f. 3), but those evoked by 5 mM glutamate declined slightly ($P = 0.0125$; analysis of variance, $F = 5.10$, d.f. 3) (Figure 3a,c). Perfusion of 1 or 5 mM L-*trans*-PDC did not enhance glutamate-evoked responses (Figure 3b,c). In fact, depolarizations by 20 mM glutamate were significantly reduced with 5 mM L-*trans*-PDC (to 78% of the corresponding response in the control group) and this effect persisted and possibly increased after removal of the glutamate uptake inhibitor from the perfusion medium. Responses evoked by 5 mM glutamate were also significantly smaller after removal of L-*trans*-PDC (Figure 3b,c).

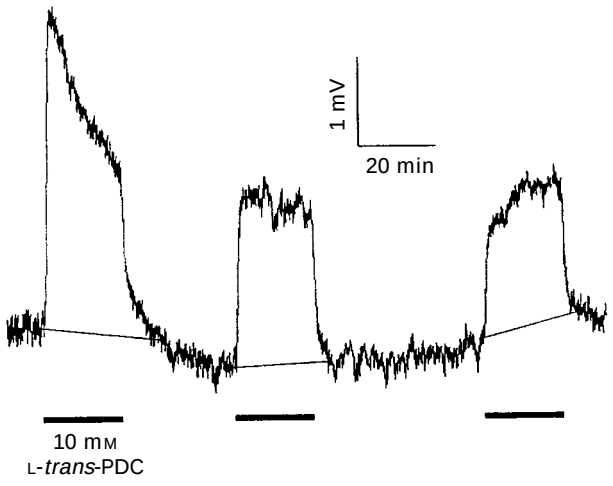

Figure 2 Representative negative shifts of the d.c. potential produced by three separate perfusions of 10 mM L-*trans*-PDC through a microdialysis probe implanted into the striatum (horizontal solid bars) (control group). With each depolarizing response, the area between the trace and the fine straight line, expressed in mV min, was used to quantify the magnitude of the responses, and for comparisons between groups (see Table 1 and data presentation and statistical analysis in Methods).

Table 1 Effect of ionotropic glutamate-receptor blockade on depolarizations evoked by application of L-*trans*-PDC (10 mM) to the rat striatum

	Amplitude of L-trans-*PDC-induced depolarization (as % of the first, control application)*	
	2nd application	*3rd application*
Control	78.8 ± 4.2	70.1 ± 5.3
MK-801, NBQX	70.0 ± 5.3	80.3 ± 4.4
NBQX, MK-801	70.9 ± 7.3	60.6 ± 5.5

L-*trans*-PDC (10 mM) was perfused through a microdialysis electrode implanted into the rat striatum during 3 separate periods of 20 min (see Figure 2). In a control group, animals were not treated with any inhibitor of ionotropic glutamate receptors. In a second group, animals received MK-801 (2 mg kg^{-1}, i.v.) 15 min after the first L-*trans*-PDC application, and NBQX (30 mg kg^{-1}, i.p.) 15 min after the second. This order was reversed in a third group. Values are mean $\pm$ s.e.mean ($n = 5$ to 7) of the area under the curve (change in d.c. potential vs time), expressed as % of the first L-*trans*-PDC-evoked response within each group (see Figure 2). There was no significant difference between groups (Mann-Whitney test).

Discussion

Methodological considerations

No attempt was made to calculate the true extracellular concentrations of glutamate from dialysate levels and the microdialysis efficiency (i.e. extraction fraction or recovery) because *in vitro* recovery (around 20%, with 2 mm AN69 Hospal Medical fibre at 1 μl min^{-1}) does not correspond to that determined *in vivo* (Kehr, 1993), especially when the uptake and/or release of the compound under study is altered (Smith & Justice, 1994).

Advantages and limitations of L-trans-*PDC*

L-*trans*-PDC is one of the most useful tools as an inhibitor of high-affinity glutamate uptake because it has virtually no action on ionotropic glutamate receptors (Bridges *et al*., 1991), but recent studies have revealed two potential pitfalls: (i) L-*trans*-PDC is a competitive, transportable inhibitor of glutamate uptake (Griffiths *et al*., 1994); and (ii) it may have agonistic actions on metabotropic glutamate (mGlu) receptors, particularly at high concentrations (Miller *et al*., 1994).

The first drawback (i.e. competitive substrate) implies that L-*trans*-PDC is carried through the glutamate transporter systems into both neurones and astrocytes when it is applied to *in vitro* preparations or directly into the brain (Rauen *et al*., 1992). As with glutamate, L-*trans*-PDC transport is associated with Na^+ influx and K^+ efflux (Kanner & Bendahan, 1982), depolarization of the cellular membrane because this transport system is electrogenic (Brew & Attwell, 1987; Sarantis *et al*., 1993) and, consequently, increased energy demand due to Na^+, K^+-ATPase activation (Eriksson *et al*., 1995). In addition, by entering the cells, L-*trans*-PDC provokes an efflux of glutamate by carrier reversal (i.e. heteroexchanges; Waldmeier *et al*., 1993; Griffiths *et al*., 1994).

With regards to agonistic actions on mGlu receptors, L-*trans*-PDC was found to stimulate phosphoinositide (PI) hydrolysis, and inhibit isoprenaline stimulated cyclic AMP ac-

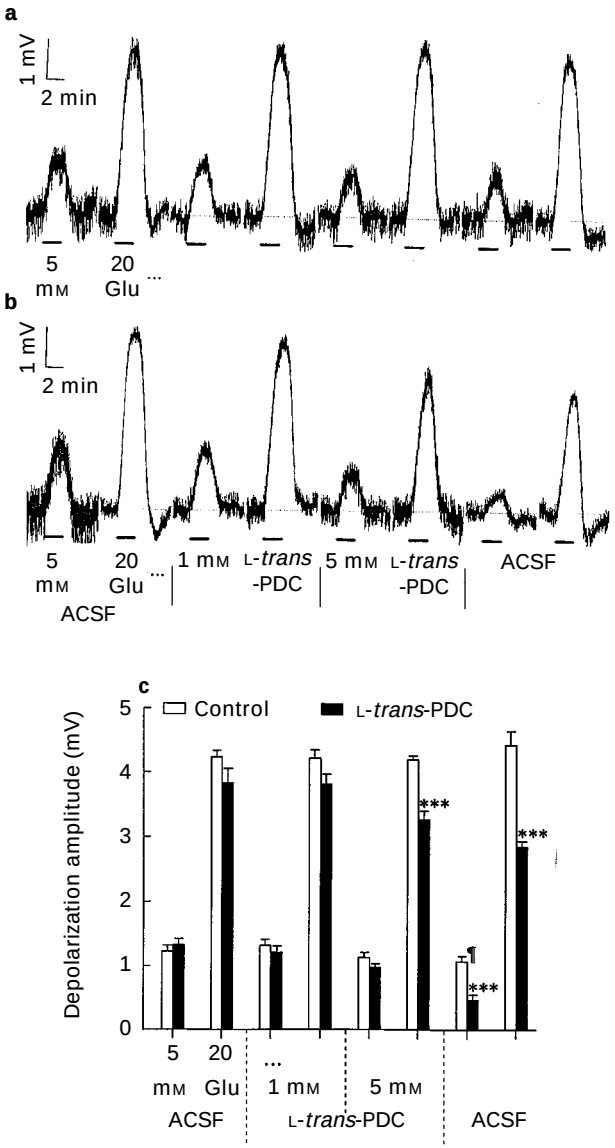

Figure 3 Effect of inhibition of glutamate-uptake by L-*trans*-PDC on depolarizations evoked by application of exogenous glutamate to the rat striatum. The d.c. potential traces show representative depolarizations produced by repeated, paired applications of 5 and 20 mM glutamate. Each sequence of responses was taken from a single experiment; (a) control; (b) co-application of 1 or 5 mM L-*trans*-PDC, and return to normal aCSF. (c) Mean ($\pm$ s.e.mean; $n=6$) of the amplitude of glutamate-evoked responses. ¶Overall effect of time on depolarizations evoked by 5 mM glutamate: $P<0.02$ (analysis of variance, $F=5.10$, d.f. 3). *** $P<0.001$, compared to the control group by Student's t test. Note that inhibition of glutamate-uptake by L-*trans*-PDC did not potentiate glutamate-evoked depolarization; actually, 5 mM L-*trans*-PDC reduced it, and this effect persisted.

cumulation, in cultured cortical astrocytes (Miller *et al*., 1994). However, there is some controversy on the origin of these effects because they could be secondary to glutamate efflux by heteroexchange, instead of reflecting a direct action of L-*trans*-PDC on mGlu receptors (Thomsen *et al*., 1994). On the one hand, the metabotropic effects of L-*trans*-PDC in cultured cortical astrocytes could not be reduced with enzymatic treatment of the cultures to remove extracellular glutamate, suggesting that these effects are not secondary to glutamate uptake inhibition (Miller *et al*., 1994). On the other hand, L-*trans*-PDC stimulated phosphoinositide (PI) hydrolysis in kidney cells expressing the mGlu1a receptor but PI hydrolysis stimulation was markedly attenuated in the presence of glutamate-degrading enzymes, and L-*trans*-PDC did not bind to the mGlu1a receptor (Thomsen *et al*., 1994). Also supporting the latter interpretation is the finding that L-*trans*-PDC stimulated PI hydrolysis in a human cell line only when the mGlu1a receptor and the glutamate-aspartate transporter (GLAST) were co-expressed (Desai *et al*., 1995).

Effects of glutamate-uptake inhibition on dialysate glutamate and basal d.c. potential

Intracerebral application of L-*trans*-PDC markedly and concentration-dependently increased extracellular glutamate levels, but these changes were associated with only minor negative shifts of d.c. potential. Indeed, a 1700% increase in the concentration of glutamate in the dialysate emerging from the parietal cortex was associated with a depolarization of around 1.7 mV, i.e. around 1/10 the amplitude of cortical spreading depression (Obrenovitch & Zilkha, 1995). Spreading depression is a propagating wave of cellular depolarization, which promotes tissue damage in stroke models and is extremely sensitive to NMDA-receptor blockade (Obrenovitch, 1995).

As the increase in dialysate glutamate produced by 10 mM L-*trans*-PDC is similar to that measured during the early stage of cerebral ischaemia (Obrenovitch, 1996), this finding does not support the notion that increased extracellular glutamate is the cause of anoxic depolarization. This is in line with previous pharmacological studies: (i) the NMDA antagonists, MK-801, ketamine and 2-amino-7-phosphonoheptanoate did not delay the occurrence of anoxic depolarization (Marrannes *et al*., 1989; Lauritzen & Hansen, 1992; Xie *et al*., 1995), although MK-801 attenuated the rapid decline of extracellular Ca^{2+} reflecting its influx into brain cells (Xie *et al*., 1995); and (ii) NBQX delayed the occurrence of anoxic depolarization, but this is a feature common to drugs attenuating the cellular membrane permeability to Na^+ (Xie *et al*., 1994; 1995), presumably a consequence of reduced energy demand (Urenjak & Obrenovitch, 1996).

Origins of depolarizations induced by L-trans-*PDC*

The following two features suggested that the small depolarizations induced by L-*trans*-PDC might not be caused by increased extracellular glutamate: (i) L-*trans*-PDC had virtually identical concentration-dependent effects on dialysate glutamate in the hippocampus and striatum, but the associated depolarizations were around twice as large in the striatum (Figure 1b,c), despite the hippocampus being rich in glutamate receptors (Nakanishi, 1992); and (ii) the rapid onset of depolarization with L-*trans*-PDC (Figure 2) contrasted with the progressive increase of dialysate glutamate levels (Figure 1a). The demonstration that L-*trans*-PDC-induced depolarizations were not reduced by MK-801 and/or NBQX rules out the involvement of NMDA- and AMPA/kainate-receptors.

These small depolarizations presumably reflect the transport of L-*trans*-PDC into the cells with subsequent depolarization of the cellular membrane (see above, Advantages and limitations of L-*trans*-PDC). The fact that the negative shift of the d.c. potential peaks shortly after the first application of L-*trans*-PDC (Figure 2), i.e. when the gradient of the drug across the cellular membrane is maximum, supports this interpretation. Reciprocally, the 2nd and 3rd applications of L-*trans*-PDC produced smaller depolarizations than the first, possibly because L-*trans*-PDC, remaining within the cells from the former application(s), attenuated its transmembrane gradient when the drug was added again to the perfusion medium.

Failure of L-trans-*PDC to potentiate glutamate-evoked depolarizations* in vivo

We have no explanation for the slight, but significant, decline of the 5 mM glutamate-evoked responses in the control group (Figure 3c). This finding was unexpected, especially because the amplitude of depolarization evoked by perfusion of 200 μM

NMDA tended to increase with repeated NMDA stimuli (Urenjak *et al*., 1997a,b).

The lack of potency of glutamate to depolarize or damage nerve cells in various preparations (Rosenberg & Aizenmann, 1989; Garthwaite *et al*., 1992; Obrenovitch *et al*., 1994) is generally interpreted as reflecting the potent uptake of glutamate from the extracellular space. However, glutamate-uptake inhibition by L-*trans*-PDC did not enhance glutamate-evoked responses (Figure 3). In fact, glutamate-induced depolarizations were slightly smaller with 5 mM L-*trans*-PDC and this effect persisted, and possibly even increased, after removal of the glutamate uptake inhibitor from the medium. This unexpected attenuation of glutamate-evoked responses may result from L-*trans*-PDC being transported through the glutamate carrier, producing marked alterations of the gradients of major cations across the cellular membrane, and ultimately leading to activation of Na^+,K^+-ATPase and acidosis, both capable of attenuating glutamate-evoked depolarization (Thompson & Prince, 1986; Traynelis & Cull-Candy, 1990; Urenjak *et al*., 1997b).

In conclusion, these *in vivo* data suggest that very high *extracellular* glutamate concentrations are necessary to produce excessive ion flux through glutamate-operated channels (Clements *et al*., 1992; Obrenovitch *et al*., 1994), and that inhibition of glutamate uptake is not sufficient for inducing marked depolarization, such as spreading depression or anoxic depolarization. In addition, perfusion of up to 100 mM L-*trans*-PDC for 2 h, or 25 mM L-*trans*-PDC for 4 h, through a microdialysis probe implanted in the rat striatum did not produce any significant neuronal damage, as assessed by changes in choline acetyltransferase and glutamate decarboxylase (Massieu *et al*., 1995). These results, together with *in vivo* studies of spreading depression (Obrenovitch & Zilkha, 1995) and drug-induced seizures (Obrenovitch *et al*., 1996), challenge the notion that high extracellular glutamate may be the key to excitotoxicity in neurological disorders. Impaired uptake and excessive release of glutamate may contribute to some neurological disorders (Rothstein *et al*., 1996), but deleterious consequences may be restricted to synapses (i.e. not by spreading through the interstitial fluid), and other abnormalities of glutamate-receptor-mediated transmission are likely to be involved (Obrenovitch & Urenjak, 1997).

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